Regulation of osteoblast and osteoclast differentiation by c fos and #MU#sx transcription factors

Beedles, Karen Elizabeth

Regulation of osteoblast and osteoclast differentiation and function by c-Fos and Msx transcription factors

A thesis submitted for the degree of Doctor of Philosophy at the University of London

2002

Karen Elizabeth BEEDLES

Department of Craniofacial development
King's College London

Abstract

Bone cell differentiation and remodelling are controlled by hormones, growth factors and specific transcriptional regulators. This thesis aims to investigate the role of two transcription factors, c-Fos/AP-1 and Msx-1/2 in osteoclast and osteoblast differentiation using *in vivo* and *in vitro* approaches.

c-Fos has been shown previously to be essential for osteoclast differentiation and is overexpressed in osteoclasts of Paget's disease. To investigate the role of c-Fos in osteoclasts, transgenic mice were generated where c-*fos* was overexpressed in osteoclasts using the TRAP promoter. Several TRAP-c-*fos*LTR transgenic founders were generated which developed severe bone remodelling lesions with eventual tumour formation. Histological and *in situ* expression studies showed abundant osteoclasts within these lesions which expressed c-Fos, in addition to the anti-apoptotic gene Bcl-2. These features are reminiscent of Pagetic osteoclasts and suggest that these mice are useful for studying bone remodelling disorders.

The effects of c-Fos on osteoblasts was next investigated *in vitro* using a well-defined inducible expression system. Stable MC3T3-E1 osteoblastic subclones expressing a tetracycline-regulatable c-*fos* gene demonstrated that exogenous c-Fos appeared to augment the proliferation induced by BMP-2, and inhibited BMP-2-induced alkaline phosphatase activity during differentiation. Moreover, ectopic c-Fos expression in these cells stimulated apoptosis induced by serum withdrawal and Etoposide. This apoptosis was not effectively blocked by the caspase inhibitors Z-VAD-fmk and DEVD-CHO, but was blocked by the cell cycle dependent kinase (CDK) inhibitor, Roscovitine, and ectopic Bcl-2 or $p21^{WAF1,CIP1,SDI1}$ expression. These results may provide a novel link between growth control and apoptosis in osteoblasts, such that under environmental stress, c-Fos may drive cell cycle progression and render the cell susceptible to apoptosis.

Finally, the regulation of *Msx2* by osteotropic factors using two reporter gene constructs was analysed in osteoblastic cells. PTH showed no regulation of *Msx2* expression, however, small increases were observed with BMPs. The expression of *Msx1*

during bone development was also analysed in tissues from *Msx1-lacZ* transgenic mice. LacZ expression was detected in mineralising tissues of the foetus and neonate, but no LacZ expression was observed after birth.

Taken together, these data further delineate the functional roles of these transcription factors in bone development and bone disease. Importantly, these studies provide a role for c-Fos in osteoblast apoptosis and osteoclast function, and may serve as a model for c-Fos overexpression in Paget's disease.

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PUBLICATIONS

Beedles, K.E., Sharpe, P.T., Wagner, E.F. and Grigoriadis, A.E. (1999) A putative role for c-Fos in the pathophysiology of Paget's disease. *J Bone Miner Res*, **14** (*Suppl 2*), 21-8.

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Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at this or any other university or institute of learning.

Acknowledgements

I am truly grateful to Agi Grigoriadis for his close supervision. Additional thanks go to earlier supervision by Paul Sharpe of Craniofacial Development, KCL and Jon Beresford from the University of Bath (Bath Institute of Rheumatic Diseases), and all of my colleagues I have worked with during the period of my studentship, in particular to Andy Sunters and David Thomas, and to Nadeem Faruque who skilfully generated transgenic mice for this project. I would also like to thank Eli Lilley and Prof. Murray Mickel for their financial support.

On the personal front, I would like to thank Carl for his endless support and encouragement when confidence was lacking and for having to sacrifice some of his own goals so that I could pursue a Ph.D., to thank Debbie and Imelda who encouraged me to commence this study, and finally thank my parents for bringing me into this world and supporting (but not pushing) me through all of my achievements so far.

Finally, I wish to dedicate this thesis to my (and Carl's) two, yet to be born, babies and hope they develop a yearning for knowledge and nature as I have.

1. Introduction

1.1 Bone

Bone is a dense, specialised form of connective tissue comprised of highly specialised cells, mineralised and unmineralised connective tissue matrix, and spaces that include the bone marrow cavity, vascular canals, canaliculi, and lacunae. The bony skeleton performs a number of life supporting functions including a physical support, an aid in locomotion, protection of vital organs and bone marrow, and a metabolic role as a source for mineral ions, like calcium and phosphate. In addition, bone helps to maintain the blood ion concentration and acts as a reservoir for growth factors, which can be released as required by osteoclasts, the bone resorbing cells (Marks and Hermey, 1996).

The extracellular matrix makes up the major constituent of the skeletal system, which is primarily composed of collagen fibres, calcium phosphate crystals (hydroxy-apatite) and a ground substance made up of proteoglycans, glycoproteins and other non-collagenous proteins. In addition, bone consists of several cell types: the morphologically distinct and specialised bone specific supporting cells (osteoblasts, osteoclasts, bone lining cells and osteocytes) responsible for bone metabolic activity and other cellular components including the nerves and the vascular elements which help to supply essential nutrients (reviewed in Marks and Hermey, 1996; Baron, 1999).

For all its rigidity, adult bone is by no means a permanent and immutable tissue. Rather, it is a highly active tissue which remodels and repairs itself throughout life. During development and growth, the skeleton is sculpted to produce its shape and size by the removal of bone from one site and deposition at another; a process termed modelling. In contrast, the mature, adult bone regeneration continues with the periodic replacement of old bone with new at the same location. This remodelling is a continuous turnover process, which is tightly regulated and coupled by systemic hormones and by local factors which affect osteoblast and osteoclast proliferation and differentiation. Furthermore, the structural formation of a bone is a complex, multi-stage process and the sequence of events that give rise to the skeleton must be temporally and spatially controlled in order to ensure the correct placement and proportion of bones (Olsen, 2000).

Thus, skeletal development, growth and homeostasis are all tightly controlled processes, defects in which can give rise to skeletal abnormalities and metabolic bone diseases such as osteoporosis, osteopetrosis, osteosclerosis and Paget's disease.

1.2 Bone structure

Anatomically, the skeletal system contains two basic types of bone. There are long bones which include the tibia, femur and humerus, and flat bones such as the skull bones and the ileum. Furthermore, in the mature skeleton there are two types of bone structure that can be observed macroscopically which have structural and functional differences, although microscopically they contain the same cells and the same matrix element: 1) cancellous bone (spongy or trabecular) is located in the vertebrae, in the majority of the flat bones, and in the ends of the long bones and comprises only 15% of the skeleton. This bone is made up of a network of fine interlacing plates and columns, called the trabeculae. 2) A larger proportion (85%) of bone is made up of hard, compact or cortical bone, which is located largely in the shafts of the long bones that surround the marrow cavities, and in bones of the skull, and performs mainly a mechanical and protective function (Baron, 1999).

Two types of bone, lamellar and woven bone, can also be found according to the organisation of collagen fibres. The lamellar, or secondary bone is a form of mature bone found in normal cortical bone and cancellous bone after birth. It contains collagen fibres, organised into parallel layers or sheets which are uniformly mineralised. The lamellae may be arranged concentrically around a central vascular channel forming the "Haversian system" or the "osteone" (Marks and Hermey, 1996). Prior to the formation of lamellar bone, primary, immature woven bone is produced such as during the formation of the embryonic skeleton and in a fracture callus. The collagen fibrils in woven bone interweave in all spatial directions, with irregular mineralisation. This type of bone is also found in pathological situations such as fracture healing, Paget's disease and osteogenesis imperfecta. Woven bone is remodelled and replaced by the lamellar bone to form mature bone.

External examination of a long bone shows two outer extremities (the epiphyses), an almost cylindrical tube in the middle (the midshaft or diaphysis), and a developmental

zone between them (the metaphysis). In a growing long bone, the epiphysis and the metaphysis, are separated by a layer of cartilage, the epiphysial cartilage or the 'growth plate' which is comprised of proliferative chondrocytes and cartilage matrix and is responsible for the longitudinal growth of bones. Finally, the bone surfaces at the epiphyses that help to make up the joint are covered with a layer of articular cartilage that does not calcify (Baron, 1999). The layer of cells that surround the bone, the periosteum, covers the entire outer surface except for the synovial joints. The periosteum consists of an inner osteogenic layer of osteoprogenitor cells which is important for appositional bone formation, and a dense fibrous outer layer. Similarly, the internal surface (the endosteum) is lined with layers of osteogenic cells (Baron, 1999).

The bone extracellular matrix is composed of organic matrix which is synthesised locally by bone cells, consisting mainly of collagen and noncollagenous proteins such as osteocalcin and bone sialoprotein, various inorganic salts, primarily hydroxyapatite, and cellular material and water. Type I collagen is the most abundant organic component which accounts for approximately 90% of total bone protein. Whereas, the noncollagenous proteins account for 10-15% of the total bone protein content and are thought to lend the unique properties to bone tissue. Most of these proteins are synthesised by osteoblasts, such as osteocalcin, osteopontin, bone sialoprotein and osteonectin (see section 1.3.2) (for review, see Termine and Gehron Robey, 1996; Gehron Robey, 1996; Ducy and Karsenty, 1996).

1.3 The cells of bone

Bone formation, remodelling, repair and mineral homeostasis are carried out by the coordinated actions of a number of specialised cells found in bone. Both during embryonic development and in the maintenance of bone structure of the adult, these events are dependent on the tightly regulated differentiation, function and interaction of these cell types. Three major cell types contribute to the skeleton: osteoclasts which resorb bone, osteoblasts which form the bone matrix and chondroblasts which form cartilage. In addition, there are osteocytes and bone lining cells, and also cells of the monocyte/macrophage lineage. The cells of the osteoclast and osteoblast lineages are described in more detail below.

1.3.1 Osteoclasts and bone resorption

Osteoclasts are large, multinucleated cells derived from the fusion of mononuclear haematopoietic precursor cells belonging to the monocyte/macrophage lineage (Udagawa *et al*, 1990; Chambers *et al*, 1993; Kurihara *et al*, 1990; Hattersley *et al*, 1991; reviewed by Roodman, 1996). Early studies showed that injection of normal spleen cells into osteopetrotic mice rescued this phenotype which was characterised by increased bone mass that, by definition, is caused by osteoclast dysfunction (Walker *et al*, 1973). Osteoclast precursors are recruited to the bone via circulating blood, where they proliferate and differentiate into osteoclasts through cell-to-cell interaction with osteoblastic stromal cells. Early myeloid precursors can differentiate along the osteoclastic lineage or the macrophage lineage and have a high proliferative potential. Commitment to the osteoclast lineage results in osteoclasts gradually losing their proliferative capacity. As the mononuclear cells proceed down the osteoclast lineage, they become further differentiated and eventually fuse to form immature osteoclasts. This is followed by activation where osteoclasts polarise on bone and in doing so form a ruffled membrane, whereby they resorb bone (Suda *et al*, 1996) (Figure 1.1).

Osteoclasts are highly motile cells and are usually rare in bone with only two to three per μm^3, where they can be located on most bone surfaces including endosteal bone, within the Haversian system, on trabecular bone, and occasionally on the periosteal surface beneath the periosteum. Osteoclast number increases at sites of active bone turnover, such as the metaphysis of growing bone or in pathological situations, including Paget's disease, osteoporosis, and hypercalcaemia of malignancy (Roodman, 1996).

Osteoclasts can possess up to 100 nuclei per cell, but tend to contain between 10 and 20 per cell, which are often highly variable in shape (Roodman, 1996). An ultrastructural feature of the osteoclast is its ruffled border. This is adjacent to the bone surface and is surrounded by the filamentous actin-rich sealing zone, the latter attaching tightly to the bone surface via integrins. Resorption and degradation of mineralised bone matrix occur beneath the ruffled border due to the release of proteolytic enzymes and hydrogen ions across the ruffled border into the sealing zone. Osteoclasts transport resorbed material through their cytoplasm to the extracellular environment, where it is removed by macrophages (reviewed in Boyce *et al*, 1999). Osteoclasts can be identified

by their phenotypic markers which include calcitonin receptors, band 5 isozyme of tartrate resistant acid phosphatase (TRAP), cathepsin K, carbonic anhydrase II, and the integrin $\alpha_v\beta_3$ (Suda *et al*, 1996).

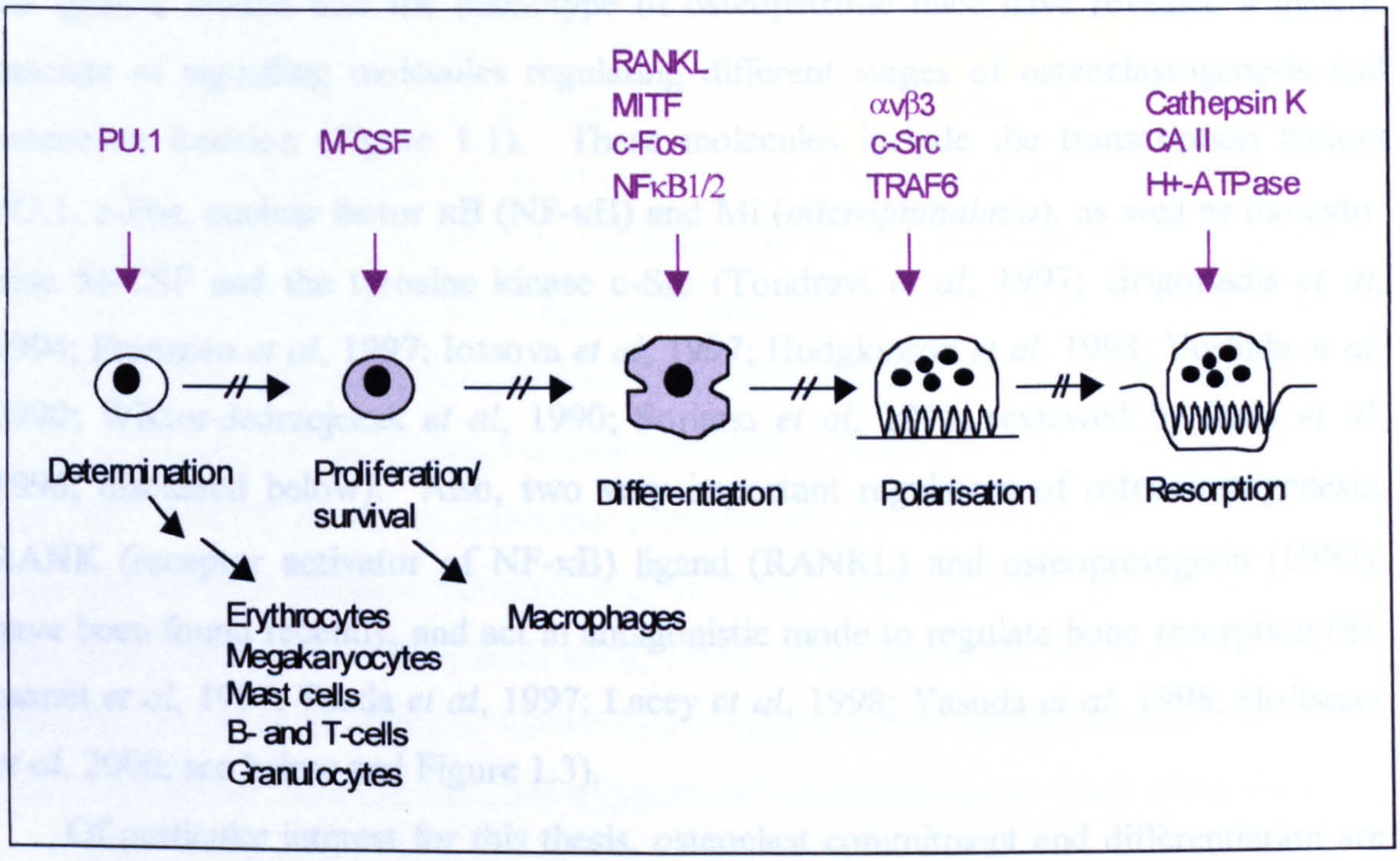


Figure 1.1 – Factors regulating osteoclast differentiation and function. Lineage diagram showing the basic stages of osteoclast differentiation from myeloid precursor to polarised, functional osteoclast capable of resorbing bone. The positioning of factors known to control this sequence of events, such as PU.1, M-CSF, RANKL, and c-Fos, are based on gene knockout studies (see section 1.3.1.1 for details). (Adapted from Teitelbaum, 2000).

1.3.1.1 Factors regulating osteoclast differentiation and function

Osteoclast proliferation, differentiation and activity are regulated by transcription factors, local cytokines and systemic hormones. Over recent years, several mouse knockout genetic studies into the phenotype of osteopetrotic mice have revealed a genetic cascade of signalling molecules regulating different stages of osteoclastogenesis and osteoclast function (Figure 1.1). These molecules include the transcription factors PU.1, c-Fos, nuclear factor κB (NF-κB) and Mi (*microphthalmia*), as well as the cytokine M-CSF and the tyrosine kinase c-Src (Tondravi *et al*, 1997; Grigoriadis *et al*, 1994; Franzoso *et al*, 1997; Iotsova *et al*, 1997; Hodgkinson *et al*, 1993; Yoshida *et al*, 1990; Wiktor-Jedrzejczak *et al*, 1990; Soriano *et al*, 1991; reviewed by Suda *et al*, 1996; discussed below). Also, two very important regulators of osteoclastogenesis, RANK (receptor activator of NF-κB) ligand (RANKL) and osteoprotegerin (OPG), have been found recently, and act in antagonistic mode to regulate bone resorption (Simonet *et al*, 1997; Tsuda *et al*, 1997; Lacey *et al*, 1998; Yasuda *et al*, 1998; Hofbauer *et al*, 2000; see below and Figure 1.3).

Of particular interest for this thesis, osteoclast commitment and differentiation are dependent on the expression of c-Fos, since c-Fos deficient mice show an early arrest in osteoclast differentiation and have increased numbers of macrophages (Wang *et al*, 1992; Johnson *et al*, 1992; also see section 1.6.3 below). Interestingly, the osteopetrotic phenotype in c-Fos knockout mice can be rescued by marrow transplantation as well as other Fos proteins, but most efficiently by Fra-1 (Grigoriadis *et al*, 1994; Matsuo *et al*, 2000; Fleischmann *et al*, 2000). Fra-1, although dispensable for osteoclast differentiation, enhances the differentiation of osteoclasts *in vitro* (Owens *et al*, 1999; Matsuo *et al*, 2000; Schreiber *et al*, 2000; Jochum *et al*, 2000; reviewed by Jochum *et al*, 2001). Furthermore, RANK activation by RANKL results in increased expression of c-Fos, and has been shown to stimulate Fra-1 expression in osteoclast precursors in a process dependent on c-Fos (Matsuo *et al*, 2000). This suggests that Fra-1 is located downstream of c-Fos in the genetic cascade controlling osteoclast differentiation. With regard to other AP-1 family members, macrophages which over-express a mutated form

of c-*jun* that cannot be activated by the kinase JNK, also show a block in osteoclastogenesis (David *et al*, 1999).

Similar to c-Fos, mouse knockouts of both the p50 and p52 subunits of the transcription factor NF-κB are also osteopetrotic due to the absence of osteoclasts (Iotsova *et al*, 1997). Mice with natural heterozygous mutations at the microphthalmia locus (*mi/mi*) develop osteopetrosis, because they fail to form multinucleated osteoclasts, a ruffled border and are defective in bone resorption (Hodgkinson *et al*, 1993). The Ets family transcription factor, PU.1 is indispensable for the development of all myeloid cells since mice deficient in PU.1 lack both macrophages and osteoclasts (Tondravi *et al*, 1997). The *op/op* osteopetrosis mouse model is deficient in osteoclasts as a result of a lack of circulating macrophage colony stimulatory factor (M-CSF) produced by stromal cells. M-CSF promotes osteoclast proliferation, differentiation, migration, chemotaxis and survival (Wiktor-Jedrzejczak *et al*, 1990; Yoshida *et al*, 1990; Lagasse and Weissman, 1997; reviewed by Suda *et al*, 1996). In addition, the non-receptor tyrosine kinase c-Src has been shown to have a role in ruffled border formation since osteoclasts from these c-Src knockout mice are incapable of resorption (Lowe *et al*, 1993; Boyce *et al*, 1992).

The recent discovery of the RANKL (also referred to as OPGL, TRANCE, ODF) and OPG families of osteoclast regulating factors has opened up a new era of molecular mechanism of the paracrine control of osteoclast development and function. OPG was found to be identical to osteoclastogenesis inhibitory factor (OCIF) cloned by Tsuda *et al* (1997) and is a soluble decoy receptor for RANKL. Targeted over-expression of *Opg* under the control of a liver-specific promoter in transgenic mice, resulted in an osteopetrotic phenotype owing to the arrest of terminal osteoclast differentiation. In contrast, targeted deletion of the *Opg* gene in mice leads to severe osteoporosis caused by increased number of functional osteoclasts (Bucay *et al*, 1998).

The ligand for OPG was later discovered revealing the molecular mechanism of OPG activity. The OPG ligand (OPGL/ODF) was found to be identical to RANKL, a TNF-like molecule, shown to bind the transmembrane receptor RANK. Similarly RANKL was also identified and termed TNF-related activation-induced cytokine (TRANCE) and osteoclast differentiation factor (ODF) by other groups (Yasuda *et al*,

1998; Wong *et al*, 1997). RANKL, synthesised by osteoblasts and stromal cells, is found in both soluble and transmembrane forms. RANKL, in conjunction with M-CSF, acts as a potent activator of osteoclastogenesis and of osteoclast function and survival *in vivo* and *in vitro*, and bypasses the need for stromal cells and 1,25-$(OH)_2D_3$ to induce osteoclast differentiation (Lacey *et al*, 1998; Yasuda *et al*, 1998; Quinn *et al*, 1998; Fuller *et al*, 1998; Burgess *et al*, 1999; Lacey *et al*, 2000). Moreover, injection of the soluble form of RANKL into mice leads to a significant increase in bone resorption (Lacey *et al*, 1998). As expected, RANKL knockout mice lack functional osteoclasts and develop severe osteopetrosis (Kong *et al*, 1999). Thus, the secretion of RANKL by activated T cells is thought to act as a systemic activator of bone resorption, leading to initiation of osteoclastogenesis and subsequent bone loss and joint destruction in auto-immune disorders (Kong *et al*, 1999).

Mice deficient in RANK, the receptor for RANKL, are devoid of osteoclasts and develop severe osteopetrosis (Dougall *et al*, 1999), and interestingly, an activating mutation in the human *RANK* gene has been found in patients with familial expansile osteolysis, a condition characterised by increased bone resorption (Hughes *et al*, 2000).

1.3.2 The osteoblast, functions and origin

Osteoblasts are the cells which not only produce bone but also act as central co-ordinators to maintain bone metabolism (Karsenty, 2000). Osteoblasts are mononuclear, cuboidal, post-mitotic cells found in contact with the bone surfaces (reviewed by Lian, 1999). Osteoblasts are polarised and have a prominent, well developed Golgi apparatus and rough endoplasmic reticulum, which indicates high metabolic activity, predominantly for the production of new bone matrix or osteoid. This osteoid is subsequently calcified by the process of mineralisation. Osteoblasts express high alkaline phosphatase activity and synthesise bone matrix proteins such as osteopontin, bone sialoprotein (BSP), and most predominantly osteocalcin, which is the only osteoblast-specific gene identified to date (Figure 1.2). Osteopontin expression appears earlier than many other matrix proteins including BSP and osteocalcin. During this stage of differentiation the expression pattern correlates with the ordered deposition of hydroxyapatite, and leads to the increased expression of other genes such as BSP and osteocalcin.

However, in the late mineralisation phase, alkaline phosphatase, osteopontin and osteocalcin appear to be down-regulated. Osteonectin, in contrast, is an osteoblast marker that can be detected early in progenitor cells and will continue to be expressed until as late as in the osteocyte stage. Characteristically, another marker of the differentiated osteoblast phenotype, is the binding of PTH (parathyroid hormone) to the PTH/PTHrp receptors and subsequent activation of adenylate cyclase, resulting in increased cAMP production. Thus, the identification of these proteins in cells is a useful tool to identify osteoblasts biochemically (reviewed by Aubin and Liu, 1996).

Osteoblasts originate from pluripotent mesenchymal stem cells of the bone marrow, whereby cells become committed to osteoprogenitor cells then further differentiate into mature osteoblasts, bone lining cells or osteocytes (Figure 1.2; Owen, 1970; Friedenstein, 1976; Aubin *et al*, 1993; Aubin and Liu, 1996; Aubin, 1998). Isolated bone marrow cells give rise to colonies of fibroblastic cells (colony-forming unit-fibroblast [CFU-F] or colony-forming cell fibroblast [CFC-F]) that, under different conditions have the potential to differentiate into a range of differentiated, connective tissue cell types including muscle, fat, cartilage and bone (e.g., Owen, 1988; Grigoriadis *et al,* 1988; Yamaguchi and Kahn, 1991; Bennett *et al*, 1991; Owen, 1985; see review by Triffitt, 1996).

Preosteoblasts lie near bone-forming surfaces where active mature osteoblasts are synthesising bone and, unlike mature osteoblasts and osteocytes, have a limited capacity to proliferate. Levels of alkaline phosphatase are lower in preosteoblasts than in mature osteoblasts and they have not yet acquired many of the protein synthesising characteristics as evidenced by a lack of a developed rough endoplasmic reticulum. An osteoblast becomes fully mature when it reaches the surface of the bone. Bone lining cells are members of the osteoblastic lineage representing a state of terminal differentiation. They are flattened cells located on the surface of bone during stages when there is no bone deposition or osteoclast resorption occurring (reviewed in Manolagas, 2000). Osteoblasts that have become terminally differentiated and encased in their own production of matrix, that has been calcified, are known as osteocytes. Osteocytes reside in lacunae and have many long cell processes which form a network of thin canaliculi, permeating the entire bone matrix and forming contacts or gap junctions with the cell

processes of other osteocytes, bone lining cells and active osteoblasts (reviewed in Noble and Reeve, 2000). Although the function of the osteocytes is debatable, they may regulate bone mass, acting as mechanoreceptor-transducers and may signal to osteoclasts to replace bone at the sites of bone which have undergone micro-damage (Noble and Reeve, 2000).

The transition from early progenitors to a fully-functional matrix synthesising osteoblast is a gradual process controlled by sequential pattern of gene expression (see below). The differences in level of expression of osteoblast-associated products and different combinations of several of the osteoblast properties is used to characterise cells at certain developmental or maturational stages (Figure 1.2). Analysis of osteoblasts *in vivo* or progression of osteoblast differentiation in model systems *in vitro*, has determined the levels of some of these osteoblast products at particular stages (reviewed in Aubin and Liu, 1996). Biochemical responses of osteoblasts to systemic hormones and growth factors have provided an indication of the osteoblastic potential in some cells. At present, there is a lack of early osteoblast stage specific markers with most of the available ones biased towards the more differentiated osteoblast and osteocytes. However, certain discrepancies have been found both *in vivo* and *in vitro* which may reflect the functional and spatial heterogeneity of the osteoblast (Aubin *et al*, 1993; Stein and Lian, 1993; Aubin and Liu, 1996; Aubin, 1998).

1.3.2.1 Factors regulating osteoblast differentiation

Although the specific regulatory mechanisms that control the differentiation from osteoprogenitor to mature osteoblast have not been defined, the responsiveness to a series of known transcription factors, such as Runx2 and growth factors, including BMPs, have been demonstrated. Genetic mouse models, either transgenic or knockout mice, have been used to determine the role of these factors in osteoblast differentiation.

Runx2, previously known as PEBP2A1/Osf2/Cbfa1, is an essential transcription factor for osteoblastic differentiation and bone formation (Ducy *et al*, 1997; Komori *et al*, 1997; Otto *et al*, 1997), being the earliest and most specific marker, and thus a master controller in bone formation. *Runx2* was found to map to the same location as *Ccd* (Cleidocranial dysplasia; Otto *et al*, 1997) and is mutated in certain human cases af-

fected with CCD (Lee *et al*, 1997; Mundlos *et al*, 1997). Of the defects in this condition, the defects in skeletal patterning and growth imply that the gene responsible is necessary for correct skeletal patterning and growth. Indeed, homozygous *Runx2* mutant mice completely lack osteoblasts, implying that Runx2 is essential for osteoblast differentiation during embryonic differentiation (Komori *et al*, 1997; Otto *et al*, 1997). Ducy *et al* (1999) also demonstrated that Runx2 was required for post-natal bone formation by differentiated osteoblasts and was shown to regulate bone matrix deposition and induce the expression of osteoblast-related genes such as osteocalcin and osteopontin (Ducy *et al*, 1999).

The transcription factors that act upstream or downstream of Runx2 remain to be identified and may not be osteoblast specific (Karsenty, 2000). BMPs enhance expression of *Runx2* in MC3T3-E1 cells (e.g., Ducy *et al*, 1997; Tsuji *et al*, 1998), whereas PTH was reported to suppress expression of *Runx2* and of its target gene, collagenase 3 (Winchester *et al*, 2000; Hess *et al*, 2001). Furthermore, several other transcription factors such as Hox-a2, Msx2, Bapx1, have been shown to regulate *Runx2* expression (Triboli and Lufkin, 1999; Kanzler *et al*, 1998).

Several other transcription factors have been identified as important regulators of osteoblast differentiation such as members of the helix-loop-helix (HLH) family of transcription factors, Twist, Id and Scleraxis (Poliard *et al*, 1995; Harris *et al*, 1995; Liu *et al*, 1997; Cserjesi *et al*, 1995 and Murray *et al*, 1992). These transcription factors can be regulated by osteotropic factors such as 1,25-$(OH)_2D_3$, glucocorticoids, TGF-β and BMPs (Kawaguchi *et al*, 1992; Ogata and Noda, 1991; Ogata, 1993; Liu *et al*, 1997). Additionally, homeobox proteins such as Dlx5 and Dlx6, and the leucine zipper and zinc finger proteins, human X-box binding protein 1 (h XBP-1) and Egr-1 (Krox-24, zif268), respectively can affect osteoblast differentiation (Simeone *et al*, 1994; Clauss *et al*, 1993 and McMahon *et al*, 1990). Moreover, inactivation of the homeobox gene, *Msx2*, causes a severe skeletal phenotype (Satakota *et al*, 2000; explained in more detail in section 1.7.1 below). The expression of the proto-oncogenes c-Myc, c-Jun, c-Fos, and related AP-1 family members (e.g ΔFosB and Fra-1) have been implicated in the regulation of bone cell function (see section 1.6 below; and reviewed in Stein and Lian, 1995; Grigoriadis, 1996; Jochum *et al*, 2001).

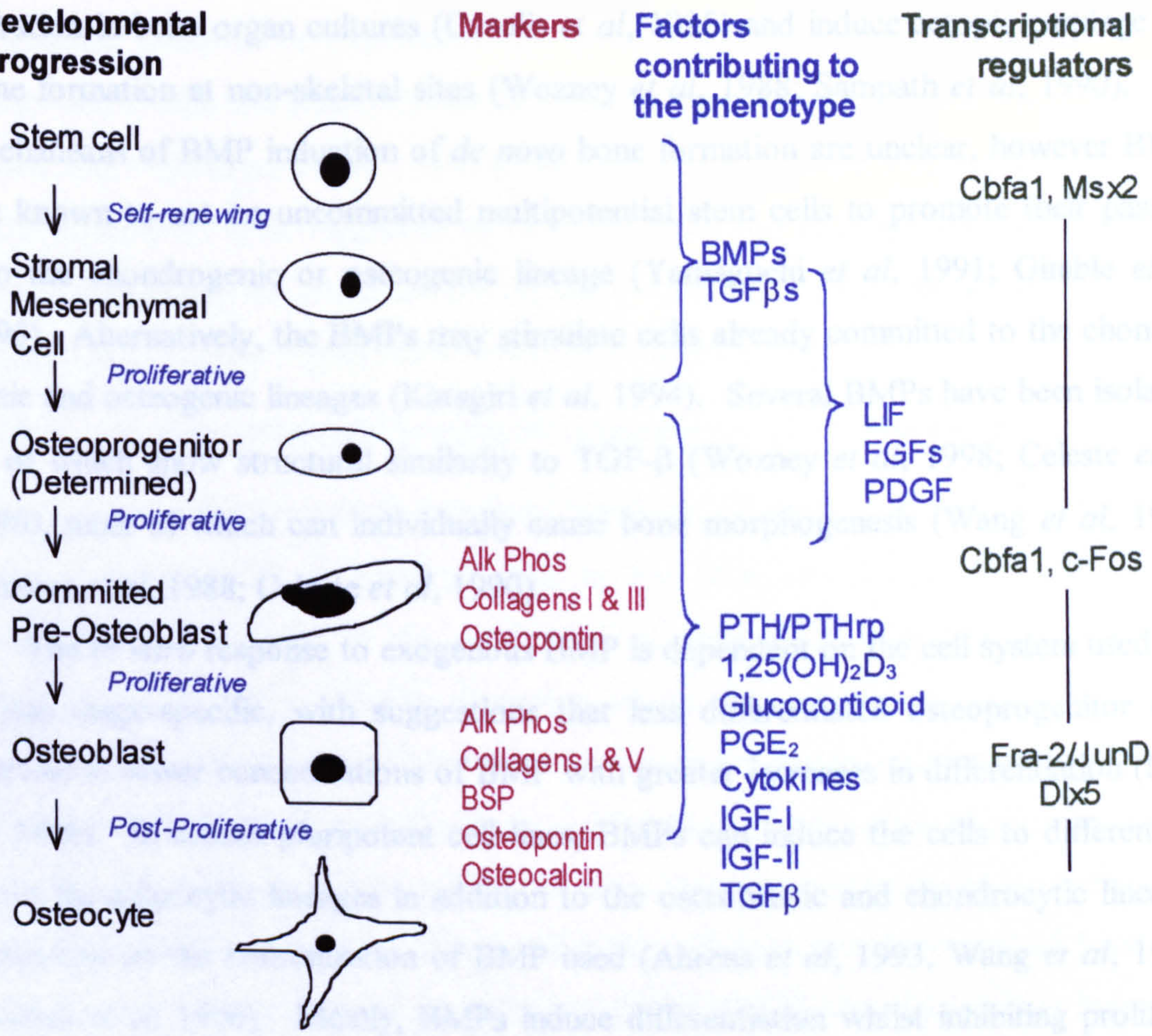


Figure 1.2 - Regulation of osteoblast lineage and differentiation. The morphological characteristics, developmental steps and many of the markers of the differentiating osteoblast are shown on the left. The factors that induce the differentiation of precursors and transcription factors which act as key regulators of bone formation are illustrated on the right. See section 1.3.2 for details. Alk Phos: Alkaline phosphatase, BSP: Bone sialoprotein. (Adapted from Lian *et al*, 1999).

Members of all the major families of growth factors, including BMPs, TGF-β, IGFs, Indian hedgehog (Ihh) and the hormones PTH, 1,25-$(OH)_2D_3$ and oestrogen have been implicated in the control of osteoblast differentiation. Bone morphogenetic proteins (BMPs) are glycoproteins, which are members of the TGF-β superfamily of

growth factors, capable of inducing new bone formation *in vivo* (Urist *et al*, 1984; reviewed by Wozney, 1993; Hogan, 1996). BMPs have been shown to promote cell proliferation in bone organ cultures (Canalis *et al*, 1985) and induce ectopic cartilage and bone formation at non-skeletal sites (Wozney *et al*, 1988; Sampath *et al*, 1990). The mechanisms of BMP induction of *de novo* bone formation are unclear, however BMPs are known to act on uncommitted multipotential stem cells to promote their passage into the chondrogenic or osteogenic lineage (Yamaguchi *et al*, 1991; Gimble *et al*, 1995). Alternatively, the BMPs may stimulate cells already committed to the chondrogenic and osteogenic lineages (Katagiri *et al*, 1994). Several BMPs have been isolated, all of which show structural similarity to TGF-β (Wozney *et al*, 1998; Celeste *et al*, 1990), most of which can individually cause bone morphogenesis (Wang *et al*, 1988; Wozney *et al*, 1988; Celeste *et al*, 1990).

The *in vitro* response to exogenous BMP is dependent on the cell system used and is also stage-specific, with suggestions that less differentiated osteoprogenitor cells respond to lower concentrations of BMP with greater increases in differentiation (Li *et al*, 1996). In certain pluripotent cell lines, BMPs can induce the cells to differentiate down the adipocytic lineages in addition to the osteoblastic and chondrocytic lineages depending on the concentration of BMP used (Ahrens *et al*, 1993, Wang *et al*, 1993, Asahina *et al*, 1996). Mostly, BMPs induce differentiation whilst inhibiting proliferation, but a minority of cell types experience accelerated proliferation. Studies on osteoblasts and chondroblast cell lines or primary cultures showed that BMPs can stimulate the expression of alkaline phosphatase, and osteocalcin, promote collagen synthesis, produce a PTH response, induce the formation of mineralised nodules and indirectly induce *Runx2* expression (Vukicevic *et al*, 1989; Sampath *et al*, 1992; Theis *et al*, 1992; Asahina *et al*, 1993). Not all BMPs induce an osteoblast phenotype *in vitro*, for example BMP-12 and -13 only induce myoblast differentiation in C2C12 cells (Inada *et al*, 1996). In addition, BMP-2 has been shown to have a stimulatory effect on osteoclast differentiation and resorption, although this may occur by indirect stimulation via stromal cells (Kanatani *et al*, 1995).

TGF-β can control the steady state level of osteoblast differentiation *in vivo* and has been shown to inhibit *Runx2* expression in cultured osteoblasts (reviewed in Ducy,

2000). However, the mechanism of how TGF-β regulates osteoblast differentiation is debatable. TGF-β can promote the expression of most differentiated phenotypes, such as alkaline phosphatase expression and extracellular matrix synthesis (reviewed in Bonewald, 1996), and is also known to stimulate bone formation when injected *in vivo* (Noda and Camilliere, 1989). TGF-β may regulate the further differentiation of committed precursors cells, suggested by its ability to induce cartilage and bone formation when it is administered under the periosteum (Joyce *et al*, 1990), but not in the *de novo* production of bone at non-skeletal sites (Centrella *et al*, 1994).

1.3.2.2 Model systems of the osteoblast phenotype and osteogenesis.

In vitro models of osteogenesis have been developed whereby long-term culture of bone marrow stromal cells or calvarial cells in the presence of ascorbic acid and β-glycerophosphate results in the production of mineralised bone-like nodules (e.g., Beresford *et al*, 1993). Nodule formation allows the complete study of the distinct stages of proliferation, extracellular matrix development and maturation and finally mineralisation. Primary marrow cell culture also allows the evaluation of gene expression related to commitment of stem cells to the osteoblast lineage (reviewed by Aubin *et al*, 1998). In addition, a number of osteoblast-like cell lines have been derived from normal bone. For example, rat (RCJ) and mouse (MC3T3-E1) calvarial cell lines have been shown to develop mineralised nodules and express osteoblast markers similar to osteoblast from primary calvarial cultures (Grigoriadis *et al*, 1988; Sudo *et al*, 1983).

As an alternative to using primary cells, the immortalisation of cells of the osteoblast lineage either by derivation of clonal cell lines from spontaneous tumours or the introduction of various adenoviruses have allowed the study of particular stages of osteoblast differentiation. For example, the most widely used osteosarcoma osteoblast-like cell lines, UMR-106, ROS 17/2, ROS 17/2.8, SaOS, TE-85, MG-63 demonstrate a varied osteoblast phenotype including PTH-responsiveness, expression of osteoblastic markers and formation of mineralised tissue *in vivo* (see review by Majeska, 1996). Rat calvaria or human bone cells have also been immortalised by transfection with large T antigen of SV-40, e.g., RCT-1 or RCT-3 and HOBIT, respectively and also show osteoblastic properties. Similarly the hFOB cell line was derived by immortalisation of

human fetal bone cells using a temperature sensitive mutant of the SV-40 T antigen and selectively supports proliferation or post-proliferative phenotypic gene expression (Majeska, 1996).

1.3.3 Formation of the skeleton and bone remodelling

The process of bone formation in embryonic development occurs by two different mechanisms. Bones of the craniofacial skeleton, as well as clavicles, derive directly from intramembranous ossification. Whereas, bones of the axial and appendicular skeleton derive from an indirect process of endochondral ossification.

The process of intramembranous ossification occurs when mesenchymal cells within a well polarised region of the embryo differentiate directly into pre-osteoblasts and then mature osteoblasts (see section 1.3.2). These cells synthesise a woven bone matrix and at the periphery of this bone a population of mesenchymal cells keep differentiating into precursors of osteoblasts to allow what is known as appositional growth of bone. Haematopoietic bone marrow is formed as blood vessels are incorporated into the woven bone; and later this bone is gradually resorbed and replaced in the adult by lamellar bone (Baron, 1999).

The essential difference between endochondral ossification and intramembranous ossification, is the presence of a cartilage template, which is best exemplified in long bone development. A population of mesenchymal cells, derived from the sclerotome and lateral plate mesoderm, first undergo division and differentiation into pre-chondroblasts which then mature into chondroblasts capable of secreting collagenous matrix (Olsen, 2000). Chondroblasts become chondrocytes as they are embedded within the gel-like cartilage matrix, where they continue to grow via interstitial growth. The chondrocytes within the cartilage become progressively larger, a process known as hypertrophy, after which they die off as the cartilage becomes calcified.

Following calcification, the cartilage is partially resorbed by selective resorption carried out by osteoclasts. Osteoblasts are recruited in to form a collar of woven bone by intramembranous ossification in the future mid-shaft area of long bones, under the perichondrium, which is then calcified. Selective osteoclastic resorption allows formation of the future haematopoietic bone marrow by facilitating the vascular invasion of

both this newly formed bone and the previously avascular embryonic cartilage. A layer of woven bone is produced by osteoblasts on top of the cartilaginous remnants, which is termed primary spongiosa. Later this woven bone is further resorbed and replaced with lamellar bone, reaching the mature state known as the secondary spongiosa (Baron, 1999).

1.3.3.1 Bone remodelling

Bone remodelling in the adult is a turnover mechanism, replacing the old bone with new and thus allowing control of mineral homeostasis (Mundy, 1999). It is a complex, dynamic process involving a number of cellular functions controlling the resorption and formation of new bone so that bone mass is maintained constant throughout adult life. The first remodelling of bone takes place during embryogenesis and is known as the first activation-resorption-formation (ARF) sequence. Once the shape of each bone is formed during embryogenesis and modelled throughout bone growth, the activity of osteoblasts and osteoclasts is co-ordinated, in a process known as coupling, to ensure the maintenance of a mineralised bone matrix in the adult bone.

Bone remodelling basically consists of two phases: bone resorption by osteoclasts followed by formation by osteoblasts, and generally, bone formation only occurs where resorption has taken place. As explained above, the signal to resorb bone is received and processed by the osteoblast, which then expresses M-CSF and RANKL to recruit and stimulate the activity of osteoclasts (Suda, 1999). In the adult bone, osteoblasts play a pivotal role in directing both when and where bone resorption will occur and require osteoclastic resorption to occur for their activation and subsequent bone formation. During the ARF sequence, osteoclasts are recruited to the site of remodelling, and become activated to resorb the bone. An intermediate phase between resorption and formation, known as the reversal phase, is distinguished by the presence of a cement line marking the limit of resorption and acts to cement together the old and the new bone. Following the mineralisation of new bone some osteoblasts become inactive bone lining cells and the bone surface enters a resting phase (Mundy, 1999).

1.3.3.2 Factors regulating bone formation and remodelling

Bone remodelling is regulated by a number of systemic hormones including PTH, 1,25-$(OH)_2D_3$, glucocorticoids, gonadal steroids, and insulin, in addition to regulation at the local level by autocrine and paracrine factors (reviewed by Mundy, 1999; Karsenty, 2000). To achieve the balanced control of bone turnover, the above hormones and factors directly affect the proliferation of undifferentiated cells, the recruitment of cells and the differentiated function of the cells involved.

In particular, PTH and 1,25-$(OH)_2D_3$ are the two principle hormones of the calcium homeostatic system, and maintain this through the stimulation of osteoclast development and regulation of calcium absorption and excretion from the intestine and kidney, respectively. PTH has a complex effect on bone formation being responsible for both the stimulation and inhibition of bone formation, depending on the continuous presence of the hormone and its association with IGF-1 (Canalis, 1989; Dempster *et al*, 1993).

The effects of PTH are mediated via the activation of transmembrane PTH/PTH related protein (PTHrP) receptor. The PTH receptor type 1 (PTHR1) mediates signalling in osteoblasts and classical second messenger pathways such as adenylate cyclase and phospholipase C. Several studies have shown that the receptor expression is associated with specific stages of differentiation, with greatest levels found in active osteoblasts lining trabecular bone.

PTH controls the resorption of bone indirectly by stimulating the osteoblast or stromal cells to increase the generation of osteoclast precursors and osteoclast maturation and fusion. PTH also stimulates the production of collagenase and plasminogen activator (Otsuka *et al*, 1984; Cowen *et al*, 1985; Hamilton *et al*, 1985), both of which are thought to control bone resorption. PTH also functions by mediating ion and amino acid transport. In osteoblasts and stromal cells, PTH (and 1,25-$(OH)_2D_3$) regulates the synthesis of collagen and alkaline phosphatase activity (Canalis *et al*, 1989; Kream *et al*, 1986; Donahue *et al*, 1988) and various cytokines such as IL-6 and IL-11 and adhesion molecules (reviewed by Manolagas *et al*, 1996). Although controversial, PTHR1 expression has been found in osteoclasts, and direct effects of PTH on osteoclast differen-

tiation and resorption have been reported. However, the effects on mature osteoclasts may be mediated through cells of osteoblast lineage (reviewed by Roodman, 1996).

The overall effects of PTH on bone are dependent on the mode of administration, which has important therapeutic implications, such as in the treatment of osteoporosis. Continuous treatment of PTH *in vitro* was shown to inhibit collagen synthesis, but intermittent exposure stimulated collagen synthesis via local production of IGF-I and hence inhibition of osteoblast apoptosis (Canalis *et al*, 1989).

1,25-$(OH)_2D_3$ has complex effects on bone formation and osteoclast function. Vitamin D receptor (VDR) deficient mice develop rickets. Their osteoclast differentiation is normal, indicating that 1,25-$(OH)_2D_3$ is not necessary for osteoclast differentiation *in vivo* (Balsan *et al*, 1986; Li *et al*, 1997; Yoshizawa *et al*, 1997). The effects of 1,25-$(OH)_2D_3$ on osteoblast differentiation is controversial. In VDR deficient mice osteoblast differentiation is normal. Whilst *in vitro* treatment of 1,25-$(OH)_2D_3$ can inhibit osteoblast differentiation and inhibit expression of *Runx2*, alkaline phosphatase activity and collagen synthesis (Ducy *et al*, 1997). In other studies, 1,25-$(OH)_2D_3$ induced osteoblast differentiation and stimulated the synthesis of osteocalcin, collagen and alkaline phosphatase (Fritsch *et al*, 1985; Manolagas *et al*, 1981; Beresford *et al*, 1984). However, the complex response of the osteoblast to 1,25-$(OH)_2D_3$ is thought to be due to species differences, cell density and the proliferation/differentiation stage of the cells.

Recently, leptin has been identified as a hormone important in controlling bone remodelling. Leptin is synthesised by adipocytes and acts to signal to the hypothalamus to control starvation and adiposity. Interestingly, mice deficient in leptin (*ob/ob*) mice or its receptor (*db/db*) have an increased bone mass (Ducy *et al*, 2000). Leptin indirectly inhibits bone formation through actions on the hypothalamus, to inhibit the mature osteoblasts which have already differentiated, with no effects observed on osteoblast number or on osteoclast differentiation or function. This highlights a central regulatory control for bone remodelling.

Local factors, paracrine or autocrine, are also involved in the process of bone remodelling. These are best represented by the growth factors embedded in bone by osteoblasts, which are released to influence these cells at a later time. In addition to factors previously described, TGF-β is found in large quantities in bone and can stimulate

or inhibit osteoblast proliferation depending on its concentration *in vitro* and cell density (Centrella *et al*, 1987). TGF-β has been shown to inhibit proliferation and alkaline phosphatase production in osteoblastic cell lines in response to PTH (Noda and Rodan *et al*, 1987; Elford *et al*, 1987). TGF-β has diverse effects on osteoclasts: via the stimulation of osteoblasts. TGF-β can reduce osteoclast formation and stimulate apoptosis, and inhibit the effect of TGF-α which is itself known to promote the proliferation of committed osteoclast precursors in combination with EGF (Lorenzo *et al*, 1986; Takahashi *et al*, 1986); in contrast, the release of TGF-β from osteoblasts, osteoclasts, or the bone matrix, can also directly augment osteoclast formation and survival in the presence of RANKL (Fuller *et al*, 2000). Similarly, epidermal growth factor (EGF) has been shown to cause proliferation of osteoblasts *in vitro* (Ng *et al*, 1983) and various prostaglandins secreted by osteoblasts cause differentiation of osteoblastic cells (Hakeda *et al*, 1987). However, both EGF and prostaglandins are also stimulators of osteoclast resorption. In addition, IGF-I and IGF-II which have been shown to stimulate both proliferation and differentiation of osteoblasts (Hock *et al*, 1988). To this end, the majority of paracrine and autocrine activity demonstrated by these factors is particularly important in the process of controlling osteoblast differentiation.

Most of the factors known to stimulate osteoclast differentiation, such as PTH, and 1,25-$(OH)_2D_3$, prostaglandins, interleukins (IL-1, 6, 11), leukaemia inhibitory factor (LIF), oncostatin M, tumour necrosis factor (TNF)-α and -β, and TGF-α were always thought to do so by binding to receptors on stromal cells or osteoblasts, and to stimulate the release of osteoclast stimulatory factors, rather than directly via receptors on osteoclast precursors (reviewed in Suda *et al*, 1999). The discovery of RANKL and previous knowledge with M-CSF provided this signalling link between osteoblast and osteoclast. Some of the aforementioned factors mediate their signalling through three signal transduction pathways in stromal/osteoblastic cells (Figure 1.3): cAMP, gp130-, and 1,25-$(OH)_2D_3$-receptor mediated pathways (reviewed in Suda *et al*, 1996; Suda *et al*, 1999). Signals mediated by cAMP are transduced by bone resorbing factors such as PTH, PGE_2, and IL-1, although the effect of IL-1 is mediated by a mechanism involving PGE2. Cytokines such as IL-11, LIF, and oncostatin M, and a complex of a soluble IL-6 receptor (sIL-6R) and IL-6, stimulate osteoclast formation through a gp130-mediated

signal transduction pathway involving Janus family of tyrosine kinases (JAK) and signal transducers and activators of transcription (STAT) family of transcription factors to activate cytokine responsive gene transcription (reviewed in Manolagas, 2000). However, osteoclasts are produced in gp130-deficient mice possibly as a result of functional redundancy (Kawasaki *et al*, 1997). Osteoclast formation induced by 1,25-$(OH)_2D_3$ is independent of the mechanism involving cAMP and gp130, being mediated through the VDR (Suda et al, 1999).

IL-6 has attracted much attention because of its involvement in several disease states characterised by increased bone remodelling and excessive focal or systemic bone resorption such as multiple myeloma, post-menopausal osteoporosis, Paget's disease, and rheumatoid arthritis. Cytokines and growth factors, such as IL-1, TNF, PDGF, and IGF-II stimulate the production of IL-6 in stromal/osteoblastic cells (reviewed in Manolagas, 2000). The excess production of IL-6 is not sufficient to induce osteoclastic resorption and the production of sIL-6R is indispensable for IL-6-induced osteoclast bone resorption in the above conditions.

In contrast, factors which show inhibitory effects on osteoclastic development and activity and promote osteoclast apoptosis include calcitonin, IL-4, IL-10, IL-18, interferon-γ, TGF-β and nitric oxide. Some evidence suggests that many of these hormones and cytokines act indirectly through osteoblasts (reviewed by Roodman, 1996; Suda, 1997). Oestrogen has also been shown to have an inhibitory effect on osteoclast development which is thought to involve indirect stimulation of osteoclast apoptosis via TGF-β (Hughes *et al*, 1996) and indirectly induces OPG synthesis with a decrease in RANK and M-CSF production, possibly through production of IL-1 and TNF-α (reviewed by Riggs, 2000).

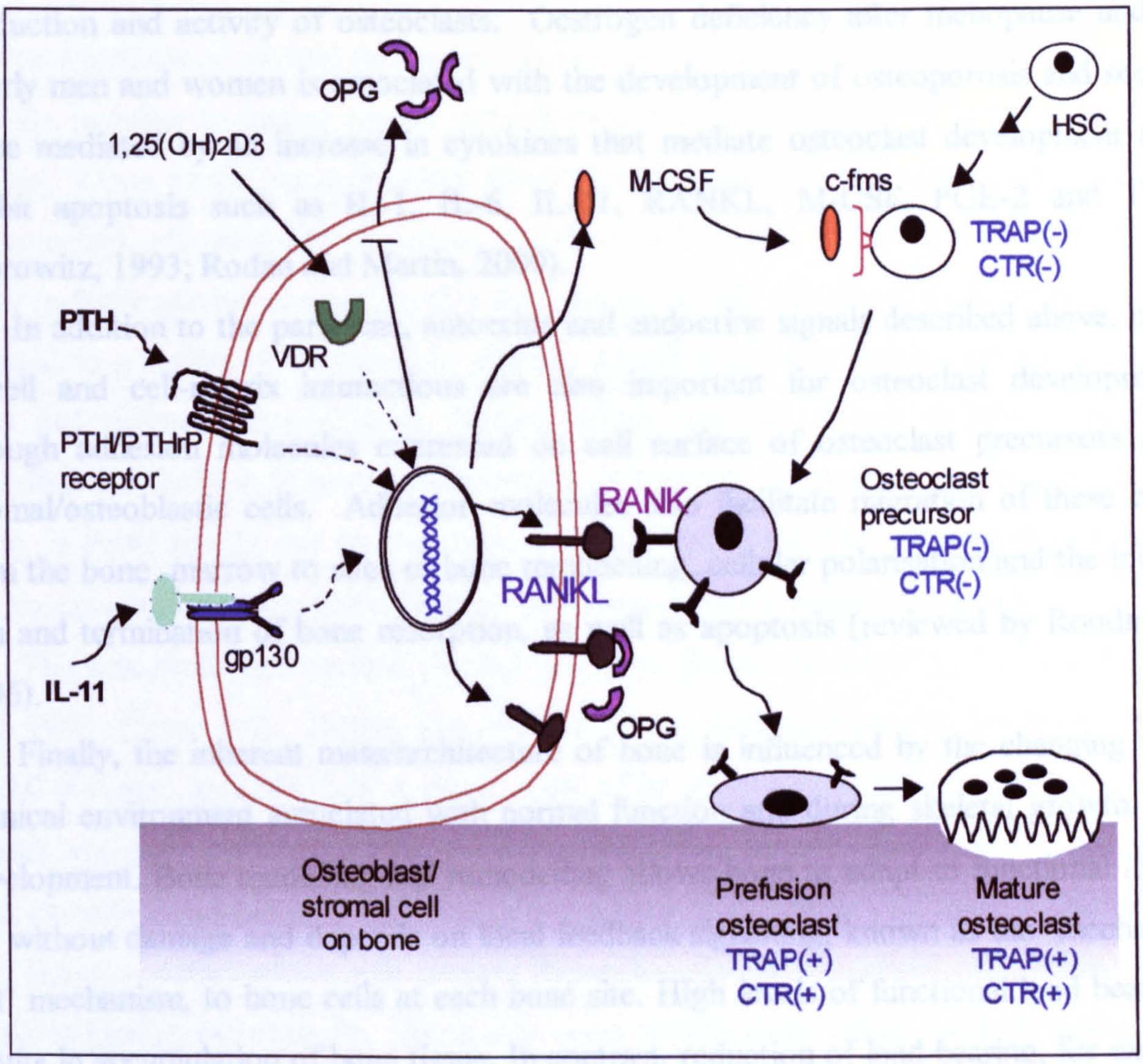


Figure 1.3 - Factors controlling osteoclast differentiation. Osteotropic factors including 1,25-$(OH)_2D_3$, PTH and IL-11 stimulate osteoclast formation via different signalling pathways mediated by VDR, PTH/PTHrP receptor, and gp130, respectively, on osteoblasts/stromal cells. Osteoblasts/stromal cells express RANKL in response to 1,25-$(OH)_2D_3$, PTH and IL-11. Osteoclast progenitors of the monocyte-macrophage lineage recognise RANKL on osteoblasts/stromal cells through cell-to-cell interaction, then differentiate into osteoclasts. M-CSF produced by osteoblasts/stromal cells is a prerequisite for both proliferation and differentiation of osteoclast progenitors. Osteoblasts/stromal cells also synthesise OPG, a process which can be inhibited by osteotropic factors, which can antagonise osteoclast formation by competing with RANK for binding to RANKL. (Adapted from Suda, 1999). HSC: haematopoietic stem cell.

Furthermore, a lack of hormones such as in the case of oestrogen, can stimulate the production and activity of osteoclasts. Oestrogen deficiency after menopause and in elderly men and women is associated with the development of osteoporosis and seems to be mediated by an increase in cytokines that mediate osteoclast development and inhibit apoptosis such as IL-1, IL-6, IL-11, RANKL, M-CSF, PGE-2 and TNF (Horowitz, 1993; Rodan and Martin, 2000).

In addition to the paracrine, autocrine and endocrine signals described above, cell-to-cell and cell-matrix interactions are also important for osteoclast development through adhesion molecules expressed on cell surface of osteoclast precursors and stromal/osteoblastic cells. Adhesion molecules also facilitate migration of these cells from the bone marrow to sites of bone remodelling, cellular polarisation and the initiation and termination of bone resorption, as well as apoptosis (reviewed by Roodman, 1996).

Finally, the inherent mass/architecture of bone is influenced by the changing mechanical environment associated with normal function and during skeletal growth and development. Bone modelling and remodelling allows bone to adapt to functional loading without damage and depends on local feedback signalling, known as the 'mechanostat' mechanism, to bone cells at each bone site. High levels of functional load bearing results in accumulation of bone tissue. In contrast, reduction of load bearing, for example with prolonged skeletal disuse results in marked osteopenia. The diminished ability to maintain bone strength in postmenopausal osteoporosis despite continued exercise is considered to be a failure of the mechanostat mechanism (reviewed by Lanyon and Skerry, 2001). Osteoblasts, osteocytes, and lining cells immediately respond to strain (and/or one or more of its derivatives such as fluid flow) and influence modelling and remodelling by influencing osteoblast proliferation, bone formation and the release of osteogenic factors such as those described above. Osteoclasts and their precursors are not well located to respond directly to strain in bone tissue, but their activity during remodelling is likely to be controlled indirectly osteoblasts, osteocytes, and lining cells (reviewed by Basso and Heersche, 2002).

1.4 Cell cycle regulation

The proliferation of normal cells is controlled by multiple growth-regulatory pathways that act together to ensure proper growth regulation. Disruption of normal cell cycle control is one of the most frequent alterations in tumour cells, which contributes to uncontrolled cell proliferation during tumour development. Tumour cells have to acquire multiple genetic changes before they display a fully transformed phenotype. Cells respond to a variety of extracellular signals, including growth factors, mitogen antagonists and differentiation-inducing factors, which together dictate cellular behaviour, including the decision to grow, differentiate, or undergo apoptosis. Cancer cells ignore many of these signals due to mutations in genes that control either growth-promoting (proto-oncogenes) or growth-inhibitory pathways (tumour-suppressor genes).

The eukaryotic cell cycle is divided into four phases (reviewed by Sherr and Roberts, 1995; Figure 1.4). During two of these phases, cells execute the two basic events in cell division: generation of a single copy of its genetic material (the synthetic or S phase) and partitioning of all the cellular components between two identical daughter cells (mitosis or M phase). The two other phases of the cycle - G_1 and G_2 - represent 'gap' periods, during which cells prepare themselves for the successful completion of the S and M phases, respectively. When cells cease proliferation, either due to specific anti-mitogenic signals or the absence of proper mitogenic signalling, they exit the cycle and enter a non-dividing, quiescent state known as G_0. To ensure proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one. It is likely that newly divided or quiescent cells must also pass certain checkpoints before they can enter the cycle.

1.4.1 Gene expression and cell cycle control

Cell cycle progression during the mammalian cell division cycle are controlled by several classes of cyclin-dependent kinases (CDKs). The activities of these CDKs are regulated by various mechanisms including association with cyclins, phosphorylation/dephosphorylation events, as well as association with two families of CDK inhibitors (CKIs) which prevent activation of the kinases and uncontrolled replication (re-

viewed by Muller, 1995; Sherr and Roberts, 1995; Sherr and Roberts, 1999; Figure 1.4). CDKs are constitutively expressed serine/threonine kinases which phosphorylate key substrates to facilitate cell cycle progression by the association with a specific cyclin. For example, CDK4 or CDK6 associate with D-type cyclins and CDK2 associate with cyclins E or A. Cyclins function as positive activators of the kinase activity of specific CDKs and their levels oscillate throughout the cell cycle: Cyclin D family members are expressed in G_1 phase, cyclin E at G_1-S phase, cyclin A in late G_1-S phase and G2-M phase, and cyclin B in S and G_2 phases. The sequential activation, with subsequent inactivation, of these cyclin-CDK complexes governs the progression through the cell cycle.

The classification of CKI families are based on their structure and CDK targets. The Cip/Kip (p21, p27, p57) family bind to all G_1-cyclin/CDK complexes, whereas the INK4 (p15, p16, p18, p19) family only bind to CDK4 and CDK6 (Sherr and Roberts, 1995). CKIs play key roles in the response of cells to growth-inhibitory signals, such as induction of differentiation, p53 activation (e.g. in response to DNA damage), TGFβ treatment, contact-inhibition, and senescence (Sherr and Roberts, 1995).

Cyclin-CDK complexes phosphorylate a number of specific targets, the most well-characterised being the retinoblastoma family proteins, pRB, p107 and p130 (Lipinski and Jacks, 1999). pRB becomes progressively more phosphorylated as cells progress through the G_1 phase allowing pRB to dissociate from the E2F family members (Figure 1.4). The active E2F transcription factor is then free to regulate the expression of genes required for G_1 to S phase progression (Beijersbergen and Bernards, 1996).

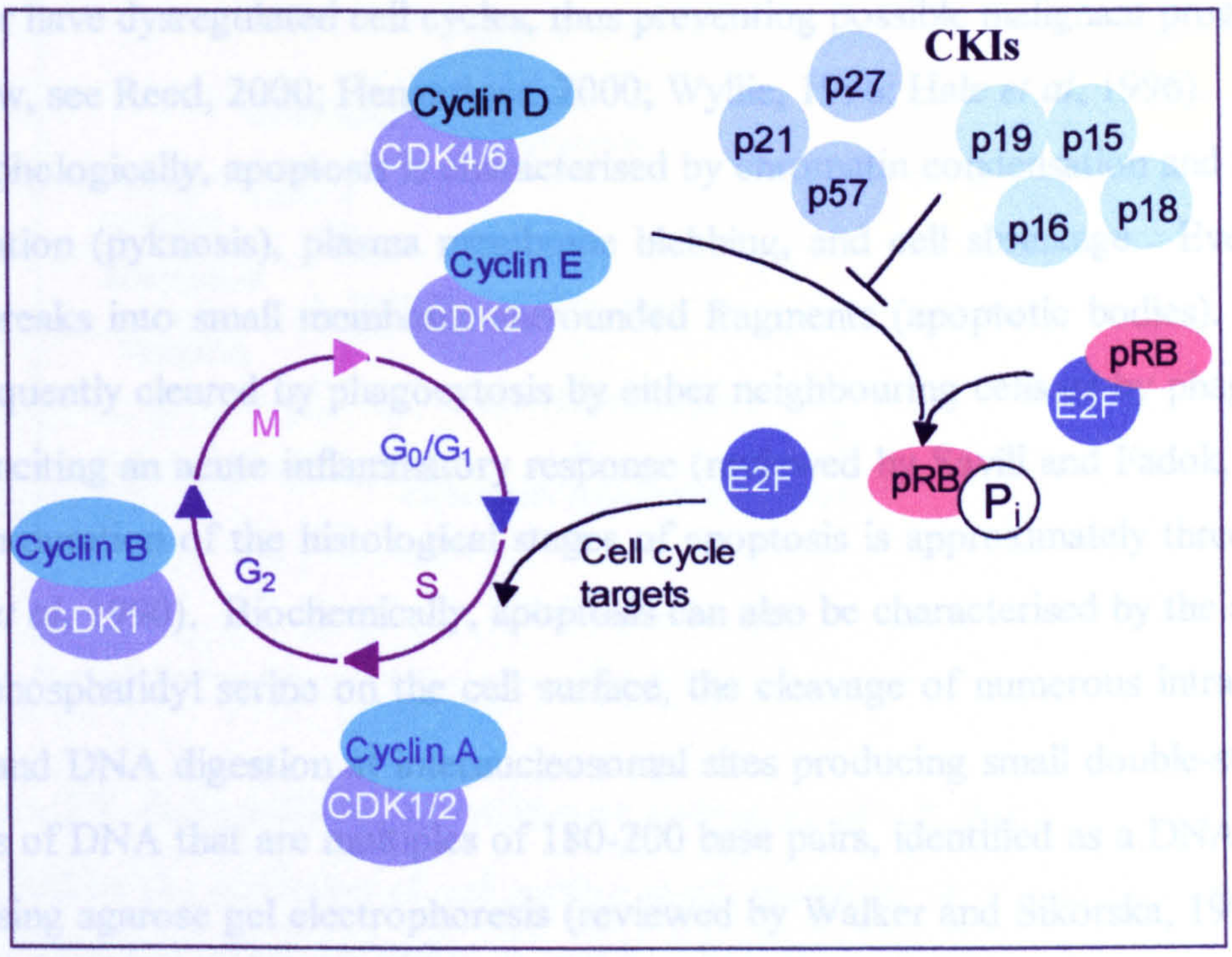


Figure 1.4 – Factors regulating cell cycle progression. The cell cycle is controlled by several classes of cyclin-dependent kinases (CDKs), which are themselves regulated by various mechanisms such as the association with cyclins and the association with two families of CDK inhibitors (CKIs): Cip/Kip (p21, p27, p57) and INK4 (p15, p16, p18, p19). Cyclin-CDK complexes phosphorylate a number of specific targets, for example the retinoblastoma family protein, pRB, which acts as a negative regulator of cell growth. pRB becomes phosphorylated as cells progress through the G_1 phase which leads to E2F dissociation. The active E2F transcription factor then regulates the expression of genes required for G_1 to S phase progression (see section 1.4 for details; adapted from Sherr and Roberts, 1995).

1.5 Apoptosis

In the past decade, there has been extensive interest in the processes of apoptosis or programmed cell death. Apoptosis is a form of cell death in which the dying cell plays an active role in its own fate under tightly controlled circumstances (Kerr *et al*, 1972) and is associated with a progressive series of morphological and biochemical changes in

the cell. Apoptosis provides a means of eliminating cells that acquire chromosomal damage or have dysregulated cell cycles, thus preventing possible malignant progression (for review, see Reed, 2000; Hengartner, 2000; Wyllie, 1996; Hale *et al*, 1996).

Morphologically, apoptosis is characterised by chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing, and cell shrinkage. Eventually the cell breaks into small membrane-surrounded fragments (apoptotic bodies). These are subsequently cleared by phagocytosis by either neighbouring cells or by phagocytes without inciting an acute inflammatory response (reviewed by Savill and Fadok, 2000). The mean duration of the histological stages of apoptosis is approximately three hours (Bursch *et al*, 1990). Biochemically, apoptosis can also be characterised by the appearance of phosphatidyl serine on the cell surface, the cleavage of numerous intracellular proteins and DNA digestion at internucleosomal sites producing small double-stranded fragments of DNA that are multiples of 180-200 base pairs, identified as a DNA ladder pattern using agarose gel electrophoresis (reviewed by Walker and Sikorska, 1997). In addition, cells undergoing apoptosis also show elevated levels of cytochrome c in the cytosol and a corresponding decrease in the mitochondria (Yang *et al*, 1997; Orrenius *et al*, 1997).

More recently, apoptosis has been defined as "the process of cell death associated with caspase activation or caspase-mediated cell death" and presumes that caspases represent its final common mechanistic pathway (Hock *et al*, 2001). In the presence of caspase inhibitors or in the total absence of caspases death is delayed (Figure 1.5) and this 'Slow cell death' may display many of the above apoptotic morphological features, suggesting that nuclear fragmentation, DNA degradation, cell shrinkage, membrane blebbing, or chromatin condensation alone are not pathognomic of apoptosis (Hock *et al*, 2001).

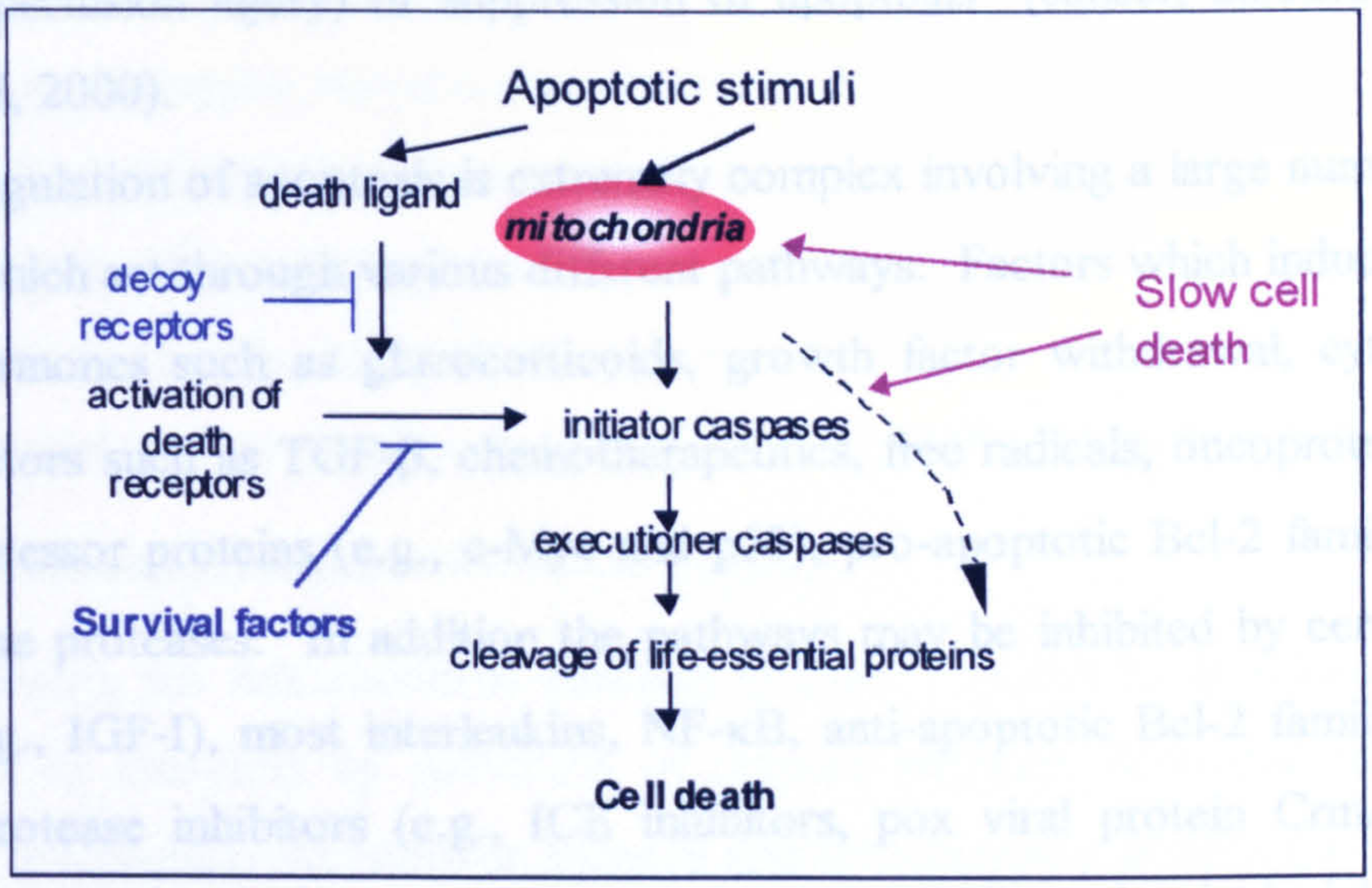


Figure 1.5 –Schematic of the different pathways of cell death. Apoptosis may be stimulated by the activation of cell surface death receptors or of mitochondria. The activation of decoy death receptors or survival factors such as in serum may inhibit these processes. In the absence of the caspase cascade, slow death may results as a consequence of cytotoxic stimulus or mitochondrial activation. (Adapted from Hock *et al*, 2001).

The above criteria distinguish apoptotic cell death from necrosis, which is cell death that occurs usually in response to physical injury. Necrosis is characterised by swelling of the cells and breaking of their plasma membranes to release proinflammatory material from the cell interior into the extracellular space. At the tissue level necrosis triggers an inflammatory response and may be an ultrafast process activated within hours, before the onset of any caspase activity.

Apoptosis plays critical roles in a wide variety of physiological processes during development including embryogenesis, organogenesis, and tissue morphogenesis (Meier *et al*, 2000) and in adult tissue homeostasis, including that of bone. Apoptosis needs to be tightly regulated, otherwise various diseases evolve because of hyperactivation of apoptosis (impaired development, neurodegenerative diseases, immunodeficiency, is-

chaemia-reperfusion injury) or suppression of apoptosis (cancer, autoimmune disorders) (Reed, 2000).

The regulation of apoptosis is extremely complex involving a large number of gene products which act through various different pathways. Factors which induce apoptosis include hormones such as glucocorticoids, growth factor withdrawal, cytokines and growth factors such as TGF-β, chemotherapeutics, free radicals, oncoproteins and tumour suppressor proteins (e.g., c-Myc and p53), pro-apoptotic Bcl-2 family members and cysteine proteases. In addition the pathways may be inhibited by certain growth factors (e.g., IGF-I), most interleukins, NF-κB, anti-apoptotic Bcl-2 family members, cysteine protease inhibitors (e.g., ICE inhibitors, pox viral protein CrmA), calcium channel antagonists, viral proteins (e.g., adenovirus E1B) and calpain inhibitors (for review, see Hale *et al*, 1996; White, 1996).

Apoptosis may occur through one or more key pathways; the two most common are the mitochondria-activated pathway, resulting in the release of cytochrome c from the mitochondria (Figure 1.6; see section 1.5.3), and the activation of one of many death receptors, such as the TNF receptor superfamily, leading to the activation of a parallel apoptotic cascade (Figure 1.7; see section 1.5.4). Pro-apoptotic agents such as growth factor withdrawal, the activation of p53, irradiation, activated oxygen, and cytotoxic drugs induce their effects through the mitochondrial pathway (reviewed in Green and Reed, 1998; Wang, 2001).

1.5.1 Caspases

The morphological and biochemical changes associated with apoptosis are caused by caspases, which are a family of intracellular cysteine proteases, that cleave their substrates at specific sites (reviewed in Earnshaw *et al*, 1999; Hengartner, 2000). Caspases are either involved in the initiation and propagation of apoptosis (e.g., caspase-2, -3, -6, -7, -8, -9, and –10; classed as homologues of *C. elegans*, *Ced-3*) or are involved in proteolytic activation (e.g., caspase-1, -4, -5; caspases related to caspase-1 [Interleukin-1 converting enzyme]). Also, caspases are subclassed as initiators (e.g., caspase-2, -8, -9, -10) or effectors or 'executioners' (e.g., caspase-3, -6, -7). Initiator caspases cleave and activate the executioner caspases as well as a variety of key proteins to irreversibly

alter cell function. For example, they interact with signalling adaptor molecules (e.g., FADD) through caspase recruitment domains (CARDs) or through death effector domains (DED) during death receptor apoptosis (Figure 1.7). The executioner caspases target substrates that induce the morphological features and DNA fragmentation of apoptosis, such as poly ADP ribose polymerase (PARP) and structural proteins for cytoplasmic and nuclear membranes (Earnshaw *et al*, 1999; Hengartner, 2000).

1.5.2 Mitochondrial-activated death pathway

In many tissues, the mitochondrial pathway (Figure 1.6) can be activated directly by apoptogens or indirectly by activation of caspase enzymes, such that mitochondrial membrane permeability transition is induced and cytochrome c is subsequently released (reviewed by Wang, 2001). The Bcl-2 family are important regulators of this process. Bcl-2 and its closely related anti-apoptotic homologues, such as Bcl-X, exert control of mitochondrial permeability and prevent cytochrome c release. In contrast, the similarly structured pro-apoptotic members of the Bcl-2 family of proteins, such as Bax and Bak, can combine and promote mitochondrial permeability via their direct effects on the mitochondria or through the inhibition of Bcl-2 (reviewed in Adams and Cory, 1998; Chao and Korsmeyer, 1998; Gross *et al*, 1999; Tsujimoto and Shimizu, 2000). Bax can homodimerise, as well as form heterodimers with Bcl-2, and the ratio of Bcl-2 to Bax is one determinant of the fate of the cell following apoptotic stimulus (Oltvai *et al*, 1993).

Once released, cytochrome c interacts with the cytoplasmic adaptor protein Apaf-1 (apoptosis activating factor) and procaspase-9 to form the apoptosome complex, which promotes the processing and cleavage of an initiator caspase, such as procaspase-9 (reviewed by Chinnaiyan, 1999). Caspase-9 then activates caspase-3, a point where this pathway converges with the death-receptor pathway (see below). The formation of the apoptosome and caspase-3 activation can be blocked by inhibitors of activation of caspases (IAPs), such as XIAP proteins (Figure 1.6), which themselves may be inhibited by the Smac protein (second mitochondrial activator of caspases [SMAC/DIABLO]) released from mitochondria (Hengartner, 2000).

A third type of Bcl-2 protein, the pro-apoptotic protein BID, provides cross-talk and integration between the mitochondrial and death-receptor pathways. BID is

cleaved by activated caspase-8 and can promote cytochrome c release (reviewed in Hengartner, 2000). Furthermore, pro-apoptotic factors exist, such as apoptosis-inducing factor (AIF), that are present in the mitochondria and released upon induction of apoptosis via caspase-independent effects, which may be necessary to ensure a swift and certain death (Susin *et al*, 1999; Wang, 2001).

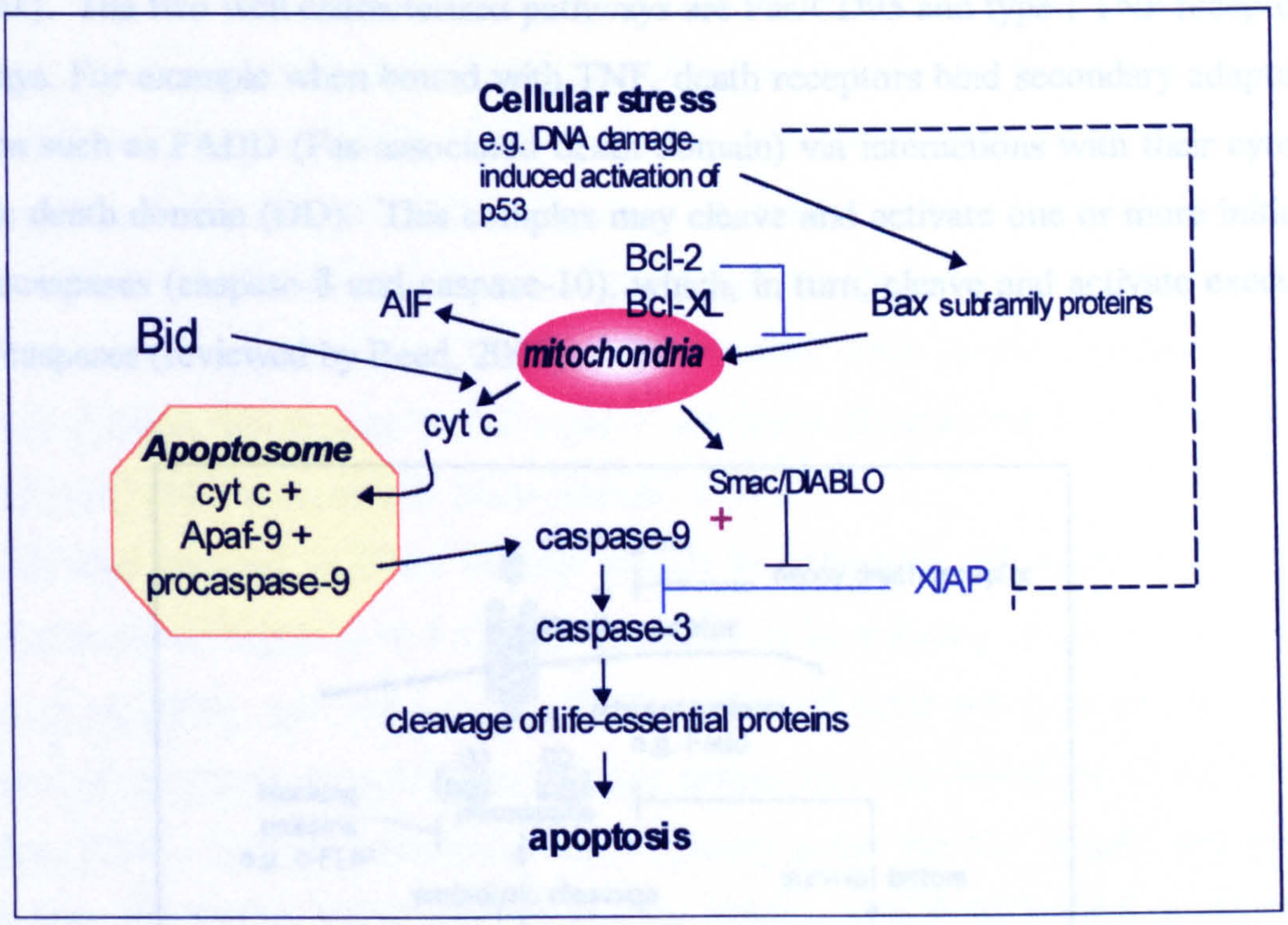


Figure 1.6 – The mitochondrial pathway of apoptosis. The formation of the apoptosome is necessary for the activation of procaspase-9 into caspase-9. Multiple feedback loops and intervention points exist to control the progression of apoptosis, for example Bcl-2 may inhibit the effect of Bax on the mitochondrial membrane. Cross-talk between the mitochondrial and death-receptor pathway is provided by Bid, a pro-apoptotic Bcl-2 family member, which causes cytochrome c (cyt c) release from the mitochondrial. (Adapted from Hock *et al*, 2001).

1.5.3 Death receptor-activated death

A parallel pathway of death induction is the activation of at least five different cell death receptors, all members of the TNF receptor superfamily (reviewed in Ashkenazi and Dixit, 1998). RANK/RANKL/OPG are included in this family of ligands and receptors and are emerging as important regulators of apoptosis in skeletal development (Hock *et al*, 2001). The two well characterised pathways are Fas/CD95 and type I TNF receptor pathways. For example when bound with TNF, death receptors bind secondary adaptor proteins such as FADD (Fas-associated death domain) via interactions with their cytoplasmic death domain (DD). This complex may cleave and activate one or more initiator procaspases (caspase-8 and caspase-10), which, in turn, cleave and activate executioner caspases (reviewed by Reed, 2000).

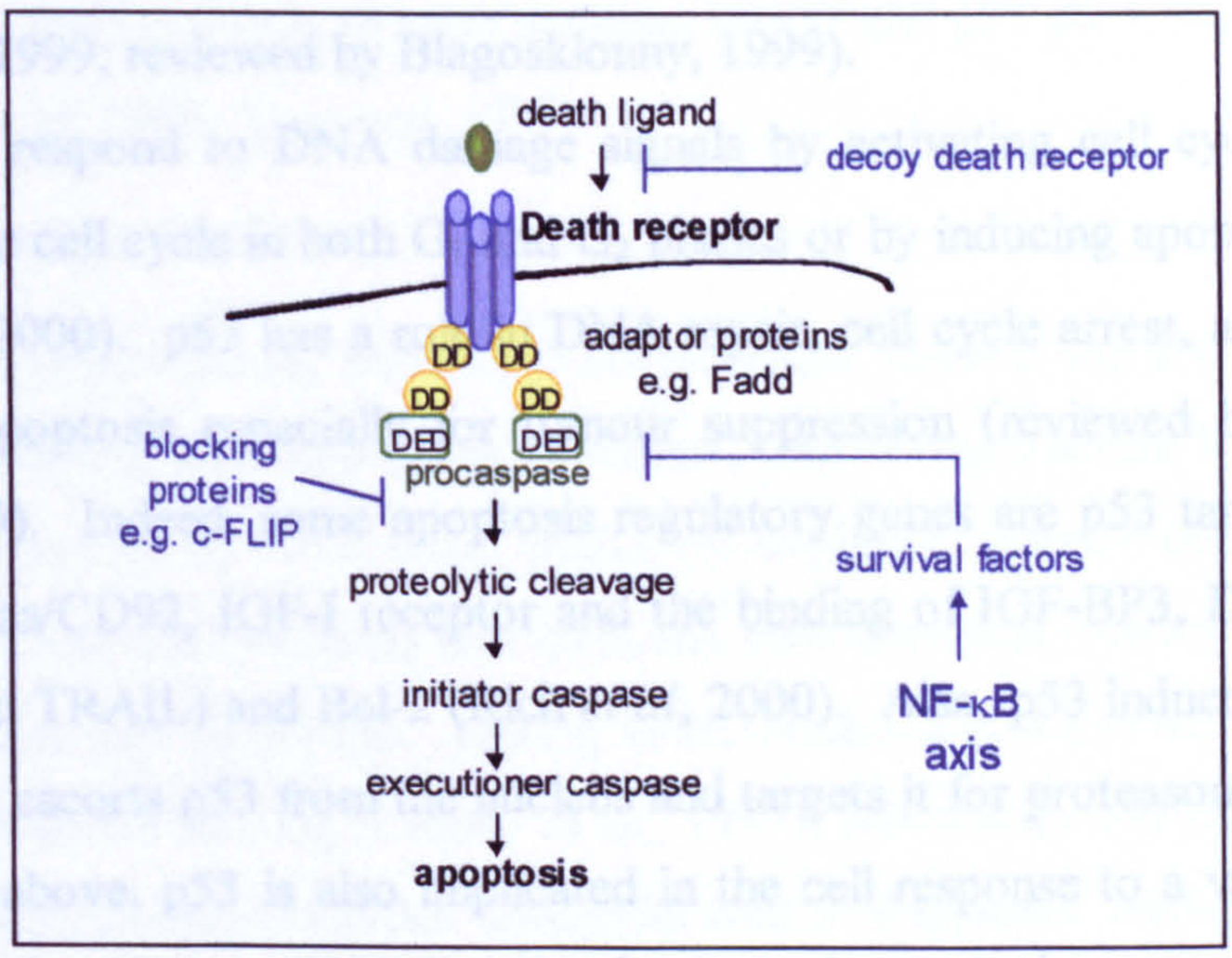


Figure 1.7 - The death-receptor pathway of apoptosis. The binding of ligands of the death-receptor superfamily, such as TNF, induce receptor clustering and formation of a death-inducing signalling complex. This recruits adaptor molecules to the death domain (DD), which then binds DED-containing pro-caspases particularly, pro-caspase-8 molecules, resulting in caspase-8 activation. This pathway may be blocked before ligand receptor binding by a decoy receptor or during intracellular steps by pathways activated to ensure cell survival. (Adapted from Hock *et al*, 2001).

1.5.4 Cell proliferation and apoptosis

Recent evidence has clearly demonstrated a link between growth and cell death. Many oncogenes and growth factors, such as c-Myc, E2F-1, and E1A, can induce proliferation and promote cellular survival, but can also induce growth arrest and apoptosis (reviewed by Evan and Littlewood, 1998).

c-*myc* was one of the first proto-oncogenes found to regulate proliferation but also induce apoptosis, such as under conditions of serum deprivation (Evan *et al*, 1992; reviewed by Prendergast, 1999). This apoptosis could be blocked by certain growth factors such as IGF-I and PDGF (Harrington *et al*, 1994). Many mitogenic oncogenes such as c-Myc, c-Fos, E2F and E1A activate apoptosis via induction of p53 through $p19^{ARF}$-dependent degradation of MDM2. This in turn leads to p53 stabilisation and apoptosis possibly by caspase-9 and Apaf-1 activation (Hermeking and Eick, 1994; Soengas *et al*, 1999; reviewed by Blagosklonny, 1999).

Cells also respond to DNA damage signals by activating cell cycle checkpoints which arrest the cell cycle in both G_1 and G_2 phases or by inducing apoptosis (reviewed by Rich *et al*, 2000). p53 has a role in DNA repair, cell cycle arrest, as well as in the induction of apoptosis especially for tumour suppression (reviewed by Oren, 1999; Vousden, 2000). Indeed, some apoptosis regulatory genes are p53 targets, and these include Bax, Fas/CD92, IGF-I receptor and the binding of IGF-BP3, DR5 (a receptor for death ligand TRAIL) and Bcl-2 (Rich *et al*, 2000). Also, p53 induces expression of MDM2, which escorts p53 from the nucleus and targets it for proteasomal degradation. As mentioned above, p53 is also implicated in the cell response to a variety of insults that do not involve obvious DNA damage, for example expression oncogenes such as c-Myc and E1A (Hermeking and Eick, 1994) and in the presence of chemotherapeutic agents (Lowe *et al*, 1993).

A number of studies have suggested a link between cell cycle events and apoptosis (Meikrantz and Schlegel, 1995). Various proteins that are involved in cell cycle control, such as p53, pRB, Cdc25, Max, c-Myc, E2F-1, and E1A, also regulate the susceptibility of cells to apoptosis (Evan and Littlewood, 1998). Like mitotic cells, apoptotic cells also have condensed chromatin, rounded morphology, and dispersed nuclear membranes and apoptosis often occurs in proliferating tissues. The phase in the cell cycle

susceptible to apoptosis seems to be from late in G_1 or early S phase. Arrest in G_0 or early G_1 suppresses apoptosis in response to a range of factors, whereas arrest in G_1 or in S phase can accelerate or potentiate apoptosis (Meikrantz and Schlegel, 1995; King and Cidlowski, 1995; Evan and Littlewood, 1998). Apoptosis appears to be linked in some cases to the aberrations in activity of CDKs (Meikrantz *et al*, 1994). The rapid up-regulation of cdc2 or CDK activity has been shown to be associated with onset of apoptosis, including induction of apoptosis following growth-factor withdrawal, TNF-α, granzyme B, Fas, γ-irradiation, heat shock and dexamethasone (Levkau *et al*, 1998; Shi *et al*, 1996; Zhou *et al*, 1998; Hakem *et al*, 1999; reviewed by Evan and Littlewood, 1998). Increased expression of the CDK activator, cdc25A, was also shown to be required for c-Myc-induced apoptosis (Galaktionov *et al*, 1996). In addition, the CKI p21 appears to play an important role in protecting cells from apoptosis (Wang and Walsh, 1996; Lu *et al*, 1998) and cells or mice deficient in p27 and p57 show increased apoptosis (Hiromura *et al*, 1999; Yan *et al*, 1997) .

1.5.5 Apoptosis in Bone

Apoptosis is important during embryonic development, skeletal maturation, adult bone turnover by modelling and remodelling processes, and during fracture healing and bone regeneration (reviewed by Hock *et al*, 2001). As explained above (section 1.3.3.2) the same growth factors and cytokines that stimulate osteoblast and osteoclast development can also influence their apoptosis. During bone formation (Jilka *et al*, 1998; Lynch *et al*, 1998) and fracture healing (Landry *et al*, 1997), osteoblasts and chondrocytes undergo a differentiation sequence that ultimately ends in apoptosis. Factors that promote osteoclastogenesis such as M-CSF and RANKL, also serve to inhibit the apoptosis of mature osteoclasts. Whereas anti-resorptive agents, such as calcitonin and bisphosphonates, are pro-apoptotic for osteoclasts but inhibit osteoblast and osteocyte apoptosis (Plotkin *et al*, 1999; Manolagas, 2000).

Little is known about the factors that control osteoblast apoptosis or the physiological importance of this process. Growth factors such as BMP-2, FGF-2 and the FGF-2 receptor may act as survival and apoptotic regulators, depending on the state of differentiation of the cells in culture (Hay *et al*, 2001; Manusukhani *et al*, 2000).

Growth factors, such as IGF-I, IGF-II, FGF2, and PDGF are required for preventing osteoblast apoptosis *in vitro*, although their role in osteoblast apoptosis remains controversial (Hill *et al*, 1997; Jilka *et al*, 1998). TNF-α and nitric acid induces osteoblast apoptosis *in vitro* (Kitajima *et al*, 1996; Damoulis and Hauschka, 1997; Jilka *et al*, 1998). TNF-α may also have a role in bone cell apoptosis *in vivo*, since it regulates the bone turnover and loss associated with ovariectomy (Kimble *et al*, 1997), and glucocorticoid and TNF-α-induced apoptosis in osteoblasts can be antagonised by IL-6-type cytokines (e.g., IL-6 and leukaemia inhibitory factor) and TGF-β (Jilka *et al*,1998; Bellido *et al*, 1998). In primary calvaria cell cultures, inhibitors of caspase-3 can block apoptosis (Weinstein *et al*, 1998) and the overexpression of Bcl-2 reduces glucocorticoid-induced apoptosis (Bellido *et al*, 1998), supporting the theory that increased osteoblast apoptosis may explain the pathogenesis of glucocorticoid-induced osteoporosis (Manolagas, 1999). Furthermore, the administration of PTH *in vivo* reduces the frequency of osteoblast apoptosis, possibly by stimulating the expression of FGF-2 and IGF-I in preosteoblasts and mature osteoblasts. These in turn induce the expression of Bcl-2 and Bcl-X_L, thereby lengthening the osteoblast's life span and so increasing bone formation. This could account for much of the anabolic response of the skeleton to exogenous PTH (Jilka *et al*, 1999; reviewed by Manolagas, 2000; Hock *et al*, 2001).

Recent research has suggested that solubilisation of bone mineral during normal bone remodelling acts as a stimulus to osteoblast apoptosis. Human bone cells exposed to phosphate ions in the culture medium, show a dose-dependent induction of apoptosis attributed to activation of the mitochondrial apoptosis pathway, which could be inhibited by inactivation of the plasma membrane sodium-inorganic phosphate transporter (Meliti *et al*, 2000). Furthermore, extracellular calcium sensitises osteoblasts to the apoptotic action of phosphate (Adams *et al*, 2001) and the anti-apoptotic property of Calbindin-D_{28k} has been attributed to inhibition of caspase-3 activity in osteoblast, in addition to its ability to chelate calcium (Bellido *et al*, 2000). Peptide fragments released from the extracellular matrix during remodelling may also play a role in apoptosis versus cell survival, for example osteonectin, fibronectin and RGD peptides may activate apoptosis in osteoblasts (reviewed by Hock *et al*, 2001).

Finally, in humans, increased osteocyte apoptosis has been correlated with sites of rapid bone remodelling and in infant calvaria (Thomkinson *et al*, 1998; Noble *et al*, 1997). Glucocorticoids and oestrogen withdrawal each promote apoptosis in osteoblasts and in osteocytes (Thomkinson *et al*, 1997; Weinstein *et al*, 1998; reviewed by Manolagas, 2000; Hock *et al*, 2001). Also, at sites of bone resorption, osteocytes undergo apoptosis and are phagocytosed by osteoclasts (Bronckers *et al*, 1996; Hughes *et al*, 1996).

1.6 The c-Fos transcription factor and bone cell function

The c-*fos* protooncogene has clearly been shown to be one of the most important regulatory genes in normal bone development and in bone disease. The c-Fos protein is a component of the AP-1 transcription factor complex that either stimulates or represses transcription of target genes by binding to AP-1 sites, leading to the regulation of transformation, cell cycle progression, differentiation, and apoptosis (reviewed in Angel and Karin, 1991; Morgan and Curran, 1991; Karin *et al*, 1997; Liebermann *et al*, 1998; Shaulian and Karin, 2001; Jochum *et al*, 2001). The AP-1 complex represents a multigene family composed of the *fos*-related (c-*fos*, *fos*B, *fra*-1 and *fra*-2) and *jun*-related (c-*jun*, *jun* B and *jun* D) genes (for review, see Angel and Karin, 1991; Shaulian and Karin, 2001). The AP-1 proteins are members of the basic region-leucine zipper (bZIP) family of transcription factors which can form homo- and hetero-dimers with each other, with activating transcription factors (ATF2, ATF3/LRF1, B-ATF), and with Jun dimerisation partners (JDP1 and JDP2) via their leucine zipper regions (reviewed by Angel and Karin, 1991; Chinenov and Kerppola, 2001). Fos proteins can only heterodimerise with Jun family members, whereas Jun proteins can form homo- or heterodimers with Jun, ATF and CREB proteins (Angel and Karin, 1991; Shaulian and Karin, 2001). More recently, Maf proteins (v-Maf, c-Maf) and the neural retina specific gene product (Nrl) have also been shown to form heterodimers with c-Jun or c-Fos; whereas other Maf related proteins such as MafB, MafF, MafG and MafK only heterodimerise with c-Fos (Shaulian and Karin, 2001; Chinenov and Kerppola, 2001). Fos dimers are not stable but can bind DNA by forming Fos/Jun complexes which are more stable than Jun/Jun complexes (Angel and Karin, 1991).

AP-1 complexes can act as both positive and negative regulators of transcription by binding to AP-1 consensus sequences (5'-TGAG/CTCA-'3), which are also known as TREs (phorbol 12-O-tetradecanoate-13-acetate [TPA] response elements), in the regulatory regions of target genes. However, Jun-ATF dimers or ATF homodimers prefer to bind to the cAMP-responsive element (CRE; 5'-TGACGTCA-3'). Overall, the AP-1 activity can be modulated at many levels, including differential expression of AP-1 proteins, dimerisation of partners and protein-protein interactions with other transcription factors, in addition to phosphorylation and stabilisation of individual members (reviewed by Angel and Karin, 1991; Karin *et al*, 1997; Shaulian and Karin, 2001; Jochum *et al*, 2001).

1.6.1 The c-*fos* gene

The proto-oncogene c-*fos* is the normal cellular homologue of the v-*fos* oncogene, the transforming gene originally identified in the FBJ and FBR-murine sarcoma viruses (MSV). The FBJ- and FBR-MSVs were isolated from spontaneous and radiation induced-osteosarcomas, respectively (Finkel *et al*, 1966; Finkel and Biskis, 1968; for review, see Verma and Graham 1987).

The c-*fos* gene is a member of the immediate early gene family, whose transcripts are rapidly and transiently induced, devoid of *de novo* protein synthesis, in many different types of cells in response to a wide range of stimuli such as serum, phorbol esters, and growth factors (reviewed by Karin, 1997). The c-*fos* mRNA has a short half-life due, in part, to the presence of a 67-basepair sequence in the 3' nontranslated region responsible for the rapid degradation of the c-*fos* mRNA (Meijlink *et al*, 1985). Removal of these sequences has been shown to be important for ensuring high, stable expression of c-*fos* mRNA and protein in the generation of transgenic animals (Grigoriadis, 1993; see section 1.6.4).

1.6.2 Function of c-Fos

Despite the early identification of AP-1 transcription factors and retroviral homologs of some of its components, the biological function of c-*fos* and other AP-1 family members are still being elucidated. The first *in vivo* indications linking Fos-related proteins to a specific biological function in bone came from the initial studies involving the isolation

of the v-*fos* gene from spontaneous bone tumours in mice and the finding that the v-*fos*-containing FBJ- and FBR-MSVs can induce osteosarcomas following injection into rodents (Finkel *et al*, 1966; Finkel and Biskis, 1968; Ward and Young, 1976). It was already known at that time that the over-expression of c-*fos* was sufficient to induce transformation of immortalised rat fibroblasts (Miller *et al*, 1984). Also the bZIP region of v-Fos (and hence that of c-Fos) was shown to be sufficient for immortalisation and transformation of chick embryo fibroblasts, and was thought to increase binding of c-Jun to the target genes whose activation results in transformation (Jenuwien and Muller, 1987; Yoshida *et al*, 1989; reviewed by Shaulian and Karin, 2001).

In addition, the striking induction of c-*fos* expression in response to growth factors also implicates c-Fos in cell proliferation. However, the studies in c-*fos* knockout mice and ectopic expression of c-*fos in vitro* and *in vivo*, demonstrated that c-Fos is not essential for the viability, proliferation and differentiation of most cell types, with the exception of some cells that are involved in bone formation (Dony and Gruss, 1987; Ruther *et al*, 1987 and 1989; Field *et al*, 1992; Brusselbach *et al*, 1995; Brown *et al*, 1998; reviewed in Kovary and Bravo, 1991; Shaulian and Karin, 2001; Jochum *et al*, 2001). Indeed, AP-1 has been implicated in the control of proliferation for many years since its activity is induced upon mitogenic stimulation and various Fos and Jun proteins have distinct expression patterns during cell cycle progression (Angel and Karin, 1991; Kovary and Bravo, 1991; Lallemand *et al*, 1997). However, c-Fos is dispensable for normal function in most cell types, for example fibroblasts and embryonic stem (ES) cells lacking c-Fos (from c-*fos* knockout mice) proliferate normally (Field *et al*, 1992; Brusselbach *et al*, 1995; Shaulian and Karin, 2001; Jochum *et al*, 2001), indicating no growth abnormality. c-Fos is more likely to play a role in growth induction, since micro-injection of neutralising antibodies to c-Fos in fibroblasts have shown that c-Fos (and Fos B in similar studies) is required for initiation of growth following mitogenic signalling, but not the maintenance of proliferation (Kovary and Bravo, 1991). Functional redundancy may mean that other Fos proteins compensate for the absence of c-Fos. Indeed, fibroblasts deficient in both c-Fos and FosB show reduced proliferation and c-Fos$^{-/-}$ FosB$^{-/-}$ mice are about 30 per cent smaller than wild-type litter mates or the

corresponding single mutants (Brown *et al*, 1998; reviewed by Shaulian and Karin, 2001; Jochum *et al*, 2001).

The most important evidence regarding the function of c-*fos* in bone was gained from loss-of-function studies, whereby c-*fos* deficient mice lack osteoclasts and develop an osteopetrotic phenotype (Johnson *et al*, 1992; Wang *et al*, 1992). Also, c-Fos was shown to be important for the oncogenic transformation of osteoblast and chondrocytes using gain-of-function studies (Grigoriadis *et al*, 1993; see 1.6.4 below).

Evidence suggesting that c-Fos may have a specificity for bone tissue is further suggested by studies which demonstrated that, during early embryonic mouse development, c-Fos expression was found in extra-embryonic tissues (Muller *et al*, 1983), while expression during late embryonic development was restricted to the growth regions during endochondral ossification of foetal bone (Dony and Gruss, 1987; De Togni *et al*, 1988; Sandberg *et al*, 1988). In adults, c-Fos continued to be expressed in bone, as well as in the central nervous system and some haematopoietic cell types, such as macrophages, granulocytes and mast cells (Smeyne *et al*, 1992; for review, see Grigoriadis *et al*, 1993). Interestingly, c-Fos was found to regulate the expression of a number of genes which are thought to have distinct roles in bone metabolism and remodelling, for example osteocalcin, collagenase, COL1A1 and stromelysin (Schule *et al*, 1990; Schonthal *et al*, 1988; Kerr *et al*, 1988), further implicating a role for c-Fos in the regulation of bone cell function. Furthermore, in certain bone cell culture models, c-*fos* expression is induced by osteotropic hormones, such as PTH (e.g., Clohisy *et al*, 1992; Kano *et al*, 1994) and a correlation exists between *fos*- and *jun*-related gene expression and differentiation of primary fetal rat calvarial cells (McCabe *et al*, 1995; Stein and Lian *et al*, 1993).

With respect to pathological bone, expression of c-Fos is high in lesions from patients with Paget's disease and fibrous dysplasia (Hoyland and Sharpe, 1994; Candeliere *et al*, 1995), and c-*fos* expression is associated with a number of spontaneous and radiation-induced murine and human osteosarcomas (Schon *et al*, 1986; Wu *et al*, 1990). However, these studies do not demonstrate a causal role for c-Fos in these tumours and also raise the possibility that high levels of both, the c-Fos transcript and protein, may be only a consequence of tumour formation. The specificity of c-Fos-induced bone func-

tion and tumourigenesis was only demonstrated with *in vivo* studies involving transgenic and knockout mice (Ruther *et al*, 1987 and 1989; Johnson *et al*, 1992; Wang *et al*, 1992; Grigoriadis, 1996). These studies have shown c-Fos and other AP-1 components (mainly members of the Fos family) have important functions in both osteoclast, osteoblast, and chondrocyte biology (recently reviewed in Shaulian and Karin, 2001; Jochum *et al*, 2001).

1.6.3 Knockout mice lacking c-*fos* and the role of c-Fos in osteoclast differentiation

To help address the function of the c-*fos* gene during embryonic development and postnatal biology, loss of function experiments using gene targeting via homologous recombination in ES cells were used. Mice lacking c-Fos are viable and fertile. However, these mice lack osteoclasts resulting in an osteopetrotic phenotype characterised by a net increase in bone mass, as well as the absence of tooth eruption (Johnson *et al*, 1992; Wang *et al*, 1992; Grigoriadis *et al*, 1994). These results suggested that c-Fos is dispensable for embryonic development and osteoblast differentiation, despite the fact that osteoblasts are susceptible to oncogenic transformation by c-Fos *in vivo* (see 1.6.4 below). c-Fos deficient mice also show abnormalities of the haematopoietic system and lymphopenia. These abnormalities were both found to be secondary to the bone phenotype, since subsequent experiments involving transplantation of haematopoietic stem cells into lethally irradiated recipients showed that c-Fos was not necessary for the differentiation and activity of haematopoietic cells (Okada *et al*, 1994; Jain *et al*, 1994).

Using osteoclast markers (TRAP and MMP-9) it was demonstrated that c-Fos-deficient mice show a complete lacked of functional, multinucleated osteoclasts and their immediate precursors (Grigoriadis *et al*, 1994). Furthermore, the osteopetrosis could be rescued by transplantation of wild-type haematopoietic stem cells into lethally irradiated mutant mice, whereas mutant haematopoietic progenitors could not form osteoclasts in the presence of wild-type osteoblasts. These experiments demonstrated that the block in differentiation in c-Fos-deficient mice was intrinsic to the osteoclast lineage, rather than the stromal/osteoblastic cells. Furthermore these loss of function studies supported the notion that osteoclast and macrophages may share a common

progenitor cell and identified a biological function for c-Fos in osteoclast differentiation and bone remodelling, and possibly also for osteoclast-macrophage lineage determination (reviewed in Grigoriadis *et al*, 1996).

Recent research using retroviral gene transfer into c-Fos mutant osteoclast precursors *in vitro* and expression of a Fra-1 transgene *in vivo* have shown that Fra-1 can most efficiently, out of all Fos proteins, complement for the absence of c-Fos in osteoclast differentiation (Matsuo *et al*, 2000). In addition, knock-in mice generated by the insertion of the *fra*-1 gene into the c-*fos* locus resulted in a total restoration of osteoclast differentiation (Fleischmann *et al*, 2000). Structure-function analysis also demonstrated that the major C-terminal transactivation domains of c-Fos and FosB are dispensable for the rescue of osteoclast function, although not for Fra-1, which lacks a transactivation domain. In addition, ectopic Fra-1 expression stimulates osteoclast differentiation both in progenitor cell lines and primary osteoclast progenitors (Matsuo *et al*, 2000; Owens *et al*, 1999); although Fra-1 transgenic mice do not show any osteoclast abnormalities (Jochum *et al*, 2000). The role of Fra-1 in osteoclast differentiation remains unclear since osteoclast differentiation does not require the presence of Fra-1 (Schreiber *et al*, 2000). However, the osteoclast differentiation factor RANKL induces Fra-1 expression in a c-Fos dependent way, highlighting the link between RANK signalling and AP-1 expression during osteoclast differentiation (Matsuo *et al*, 2000).

1.6.4 c-Fos specific transformation of osteoblasts and chondroblasts

To further study c-Fos functions in cell differentiation and development, c-Fos was ectopically expressed in transgenic mice. These mice were shown to develop bone lesions due to the *in vivo* induction of transformation by c-Fos. Two c-*fos* transgenic mice lines have been generated in which expression of the c-*fos* gene was under the control of either the human metallothionine promoter (hMT) or the promoter region of the heavy chain of the major histocompatability complex (MHC) class I antigen, H-2Kb (H2). In addition, the 3' mRNA destabilising sequences and polyadenylation (polyA) site of c-*fos* were replaced with a 3' LTR from the FBJ-MSV to provide a polyadenylation signal for termination of the mRNA molecule and to ensure stability of the c-*fos* mRNA. These constructs were termed MT-c-*fos*LTR and H2-c-*fos*LTR, respectively.

MT-c-*fos*LTR mice developed specific lesions in the long bones as early as 2-3 weeks after birth. However, only a low frequency of these mice developed osteosarcomas after a considerably long latency period of 9-10 months (Ruther *et al*, 1987; 1989). The H2-c-*fos*LTR transgenic mice induced osteosarcoma formation especially in the areas of the distal femur and proximal tibia after 4-6 weeks after birth and were more prevalent than osteosarcomas from MT-c-*fos*LTR (Grigoriadis *et al*, 1993). By 14 weeks, these lesions rapidly developed into large calcified tumours present in virtually all bones of the body. Histological analysis of these tumours revealed that they resembled chondroblastic osteosarcomas and they were highly mineralised, containing large areas of bone lined with numerous osteoblastic cells expressing high levels of alkaline phosphatase activity. Exogenous c-*fos* expression occurred between 2-3 weeks of age in calvaria, long bones and spine indicating that expression of the transgene in bone tissue occurs post-natally and before the initial appearance of bone lesions. Northern blot analysis on tumour tissue as well as on unaffected tissues of adult transgenic mice revealed that, as well as tumour tissue, the transgene was also expressed at high levels in the heart, lung, brain and salivary glands. Furthermore, established cell lines generated from these tumours were also shown to express high levels of exogenous c-Fos as well as bone cell marker genes. When injected into nude mice, these cell lines were tumourigenic with a latency period of 2-5 weeks, some of which gave rise to osteosarcomas, expressing exogenous c-*fos* mRNA and c-Fos protein in osteoblastic cells (Grigoriadis *et al*, 1993).

The phenotype of the c-Fos transgenic mice was demonstrated to be specific to c-Fos, since transgenic mice overexpressing other AP-1 family members (e.g., Jun or FosB) do not develop any abnormalities despite transgene expression in the bone tissue. However, if a c-*jun* transgene was co-expressed in these mice, the c-Fos-induced osteosarcomas were shown to occur at a higher frequency than single c-Fos transgenic mice, although there was no difference in the time of onset of tumour formation (Wang *et al*, 1995). These tumours contained great quantities of neoplastic bone, were more remodelled, and contained large numbers of multinucleated osteoclast-like cells in comparison to tumours isolated from age matched single c-Fos transgenic mice (Wang *et al*, 1995; reviewed in Grigoriadis *et al*, 1995). The results from c-Fos/c-Jun double transgenic mice demonstrate oncogene cooperativity.

All together, these results demonstrated a specific role for c-Fos in bone tumour formation and suggested that osteoblasts are target cells for transformation by the c-*fos* transgene.

Further research showed that high c-Fos levels do not affect the differentiation potential of ES cells, since ES cells overexpressing c-Fos efficiently contribute to the development of chimeric mice (Wang *et al*, 1991). However, ectopic c-Fos expression also affects the transformation of chondroblastic cells since chimeric mice generated from c-Fos overexpressing ES cells develop chondrosarcomas with high efficiency at all skeletal sites containing cartilage (Wang *et al*, 1991). More recently, Thomas *et al* (2000) in our laboratory also showed that differentiation of chondrocytes is inhibited when c-*fos* is overexpressed during chondrogenesis *in vitro*.

Previous work from our laboratory has demonstrated that in c-Fos-induced osteosarcomas, cyclin D1 expression is specifically elevated upon initial expression of the c-*fos* transgene in the pre-malignant osteoblasts and chondrocytes and not in other transgene expressing tissues (Sunters *et al*, 1998), suggesting that modulation of cell cycle regulators may be a target of c-Fos in osteoblast and chondrocyte transformation. Indeed, cyclin D1 is a known AP-1 transcriptional target (reviewed by Shaulian and Karin, 2001; Jochum *et al*, 2001). Moreover, Dr. A. Sunters provided further *in vitro* evidence in MC3T3-E1 cells in that induction of exogenous c-Fos resulted in a reduction in cell cycle time and premature G_1-S phase transition via deregulated cyclin E/A-CDK2 dependent mechanisms (Sunters *et al*, 2000) (see also Chapters 4 and 5).

1.6.5 c-Fos and apoptosis

The c-Fos/AP-1 transcription factor has also been implicated in the control of cell death and survival, depending on the type of apoptotic insult and cell type investigated (reviewed by Karin et al, 1997; Shaulian and Karin, 2001). Specifically, many studies suggest that c-Fos may be a mediator of apoptosis, a concept based in the evidence that c-*fos* expression increases in cells undergoing apoptosis (Buttyan *et al*, 1988; Colotta *et al*, 1992; Smeyne *et al*, 1993; Hafezi *et al*, 1997; Pruschy *et al*, 1997; Inada *et al*, 1998; Wenzel *et al*, 2000; Grimm *et al*, 2001). The potential role of c-Fos in normal physiological apoptosis during development came from studies on the nervous system of c-*fos*-

*lac*Z transgenic mice (Smeyne *et al*, 1993). High expression of c-*fos* was shown to precede and correlate with neuronal cell death following treatment with kainic acid or surgical lesion, as well as in a neurodegenerative mutant. Additionally, non-neural tissues demonstrated high expression of *fos-lacZ*, these included the secondary palate, nasal septum, developing tooth germ, and embryonic heart valve cushions, all areas where apoptosis normally occurs, along with hypertrophic chondrocytes undergoing programmed cell death during bone formation and epiphyseal bone growth (Smeyne *et al*, 1993). Increased expression of c-Fos, along with the Jun family members, could also be detected following castration and upon apoptotic regression of rat secretory epithelial cells lining the ducts of the rat ventral prostate and during involution of the mammary gland at weaning, a process associated with apoptosis of milk-producing epithelial cells (Buttyan *et al*, 1988; Marti *et al*, 1994). Moreover, while c-Fos is essential for light-induced photo-receptor apoptosis in the retina, it is defective in c-*fos* deficient mice and Fra-1 can substitute for c-Fos in these mice (Hafezi *et al*, 1997; Fleischmann *et al*, 2000), although it is unclear whether this is due to a requirement for c-Fos in mediating the cell death process or whether a deficiency in c-Fos throughout development alters the expression of genes required for apoptosis.

The role of ectopic c-Fos expression on apoptosis following serum withdrawal was investigated previously in greater detail in Syrian hamster embryo (SHE) cells and a human colorectal carcinoma ($RKO^{p53+/+}$) cell line (Preston *et al*, 1996), where induction of c-Fos caused apoptosis in these cell lines. A positive role for c-Fos in modulating apoptosis was also demonstrated in serum-deprived rat fibroblasts transformed by c-Fos compared to normal fibroblasts (Smeyne *et al*, 1993). In addition, in fibroblasts from *fos-lacZ* mice, c-*fos-lacZ* expression was shown to precede apoptosis induced by Etoposide (Smeyne *et al*, 1993). Evidence for a putative role for c-Fos in apoptosis during *in vitro* osteoblast differentiation was shown when there was up-regulation of various members of the AP-1 complex including c-Fos expression at the later stages of development when apoptosis is evident (McCabe *et al*, 1995).

c-Fos has also been implicated in the apoptosis of different cell types which are dependent on distinct factors for their growth and survival such as IL-6 and IL-2-dependent murine lymphoid cells (Colotta *et al*, 1992). Also, high levels of Fos and Jun

proteins correlated with Nerve Growth Factor (NGF) withdrawal from primary rat sympathetic neurones, which are dependent on the NGF for their survival (Estus *et al*, 1994).

Interestingly, however, in contrast to apoptosis induced by serum withdrawal, research has suggested that c-Fos does not promote apoptosis during normal development and c-Fos may play a protective role. In c-Fos deficient embryos and adults, c-Jun deficient embryos, and c-Fos/c-Jun double knockout embryos, apoptotic cell death occurred normally *in vivo* in developing embryonic tissues and adult thymus and ovary, regardless of the absence of a functional c-*fos* gene (Roffler-Tarlov *et al*, 1996). The absence of c-Fos also had no effect on neuronal cell death in the spinal cord following sciatic nerve section, or in heterozygous weavers' cerebellae (Roffler-Tarlov *et al*, 1996). Moreover, primary cells isolated from c-Fos null mice can undergo normal apoptosis (Gajate *et al*, 1996).

Furthermore, c-Fos may protect cells against apoptosis under certain circumstances (e.g., Walker *et al*, 1993; Schreiber *et al*, 1995; Ivanov and Nikolic-Zugic, 1997; He *et al*, 1998). An example for a protective role for Fos against apoptosis is that induced by short-wavelength UV light. Using 3T3 fibroblasts from c-*fos* deficient mice and wild-type c-$fos^{+/+}$ cells, it was demonstrated that clonogenic survival and proliferation of cells lacking c-Fos following UV irradiation was drastically reduced and apoptotic cell death was significantly enhanced, in comparison to the wild-type c-$fos^{+/+}$ fibroblasts (Schreiber *et al*, 1995). c-Fos is also vital in prolonging the life of immature CD^{4+8+} double positive (DP) thymocytes. This research found that there was an increase in spontaneous and signal-induced cell death of DP cells in c-Fos deficient mice in response to a variety of stimuli, such as dexamethasone, ionomycin, and forskolin, but not from T-cell receptor-mediated cell death, demonstrating a protective role for c-Fos in this system (Ivanov and Nikolic-Zugic, 1997). Furthermore, the reported induction of c-*fos* in cells undergoing apoptosis is reported to be a stress response that is counterbalanced by proteasome-mediated degradation of c-Fos protein, a process stabilised by Bcl-2 (He *et al*, 1998).

Taken together, the causal role of c-Fos in apoptosis is more complex than first thought. The regulation of apoptosis by c-Fos is probably specific to the cell type and

the type of stimulus. Whilst in some cases c-Fos may stimulate cell proliferation, in others it may increase survival, and in extreme situations such as during environmental stress cell death may result.

1.7 Homeobox genes and homeoproteins

Homeoproteins are a group of helix-loop-helix transcription factors which regulate specific temporal and spatial patterns of gene expression involved in embryonic development. Each homeotic protein contains a highly conserved 60 amino acid segment known as the homeodomain. This is encoded by a 180 nucleotide DNA sequence called the homeobox. Gene expression is regulated by the binding of the homeodomain to specific DNA sequences (Krumlauf, 1994). Hox genes, first discovered in *Drosophila*, were found to regulate the development of body plan, being expressed temporally in an order which reflected their linear arrangement on a chromosome (Graham *et al*, 1989). They have since been found to be highly conserved throughout a wide variety of species. In mammals, in addition to the Hox genes that are arranged into sets of four clusters, there are other homeobox-containing genes which form different small classes depending on the sequence or association with other conserved motifs. These include the *Msx* family of homeobox genes.

1.7.1 The Msx family of homeobox genes

The vertebrate homeobox-containing genes have been identified and shown to have a sequence diverged from the Hox sequence. Sequence comparisons of these genes, which show a more restricted pattern of expression have been predicted to have more specific functions of organogenesis and cellular differentiation. The Msx genes of vertebrates are a small family of homeobox-containing genes related to the *Drosophila* gene muscle-segment homeobox (*msh*). *Msx1* (initially known as *Hox-7*) was independently identified by two groups (Hill *et al*, 1989; Robert *et al*, 1989) and was found to have significant sequence identity to *Drosophila msh* gene; with 78% sequence similarity at the nucleotide level and 92% identity at the amino acid level. Monaghan *et al* (1991) later found a second closely related murine homeobox-containing gene, *Msx2* (formally termed *Hox-8*). This protein showed 97% sequence identity in the homeobox

region of *Msx1* and an overall amino acid sequence comparison of 60%. The final member in this family is *Msx3*, but this protein shows greater identity to *Msx1* (98%) than *Msx2* in both the homeodomain and conserved flanking sequence. This family is one of the most conserved homeobox-containing gene families, with the majority of conservation lying between the homeodomains and flanking region (Davidson, 1995).

Both *Msx1* and *Msx2* are considered to function as transcription factors. They are expressed in overlapping or related patterns from early stages of tissue differentiation, in a position-related, rather than cell-type specific manner (Davidson, 1995; see Table 1 below). *In situ* hybridisation studies have shown that expression is evident in neural crest cells and their derivatives, such as branchial arches and mandibular arches, and craniofacial cartilage and membranous bone (Takahashi *et al*, 1991), developing cranial sutures in a region of bone deposition and resorption (Jabs *et al*, 1993; Kim *et al*, 1998; Liu *et al*, 1995), the developing mandible and tooth (MacKenzie *et al*, 1991; Jowett *et al*, 1993), and the apical ectodermal ridge (AER) and underlying mesenchyme of the limb bud (Davidson *et al*, 1991). The expression pattern of *Msx1* and *Msx2* have been associated with epithelial-mesenchymal interactions in organogenesis (Davidson *et al*, 1991; Takahashi *et al*, 1991; Robert *et al*, 1991; Jowett *et al*, 1993). In addition, *Msx2* has been implicated in the control of apoptosis in limb development and rhombencephalic neural crest cells by regulating the expression of BMP4 (Ferrari *et al*, 1998; Graham *et al*, 1994).

Mice deficient in *Msx1*, resulting from targeted gene disruption, die in the immediate post-natal period. These mice exhibit dental and craniofacial development abnormalities, which include incomplete cleft in the secondary palate, the failure of tooth and alveolar bone development in the mandible and maxilla, and defects in the skull, nasal bones, and inner ear (Satokata and Maas, 1994; Lidra *et al*, 1998). However, these null mutant mice exhibit no neural tube, limb, or eye defects, despite the fact that *Msx1* expression occurs in these structures. Compensatory mechanisms may occur that enable embryos to overcome the disruption in some tissues that would normally require the expression of *Msx1*. Since *Msx1* and *Msx2* show similar patterns of expression, functional redundancy may play a key role (Catron *et al*, 1996). In humans, mutations in the *MSX1* gene have been involved in cleft palate (van den Boogaard *et al*, 2000) and tooth

agenesis (Vastardis *et al*, 1996; Hu *et al*, 1998). Moreover, *Msx1* may play a role in the terminal differentiation of several cell types such as cartilage (MacKenzie *et al*, 1991; Mina *et al*, 1995) and muscle (Houzelstein *et al*, 1999), by enhancing cell proliferation and preventing differentiation.

Table 2 - Expression of msh-like genes during development (from Davidson, 1995)

Species	Gene	Principle reported sites of expression
Mouse	*Msx1*	Uterus, cervix, vagina, uterine wall, ectoplacental cone, amnion, allantois, umbilical vein, and Rathke's pouch.
	Msx1 and Msx2	Primitive streak, somatopleural lateral mesoderm, dorsal ectoderm, neural plate, dorsal mesenchyme, dorsal region of the neural tube and of the brain (hindbrain, midbrain, and forebrain), choroid plexus, cranial neural crest cells, branchial arches, facial processes, tooth germs, eye, ear nose, vibrissae, heart, pericardium, limb bud, genital tubicle, tail and tail ridge.
	Msx3	Neural tube
Xenopus	*Msx1 and Msx2*	Dorsal mesodermal mantle, lateral plate mesoderm, neural crest, dorsal neural tube, dorsal region of brain and heart
Zebrafish	*msxA, B, C, D*	Fin buds
	msxC, D	Inner ear
Drosophila	*msh*	Segment muscles and ventral nervous system

1.7.1.1 *Msx* genes and bone

The strong expression pattern in cells at extreme ends of osteogenic fronts of calvarial sutures and in adjacent mesenchymal cells have led to the proposition that Msx2 has a

role to play in the differentiation of osteoprogenitor cells. Msx2 prevents differentiation and promotes proliferation of cells at the osteogenic fronts of calvariae, facilitating expansion of the skull and closure of the suture (Liu *et al*, 1995; Liu *et al*, 1999). High levels of *Msx2* transcripts have been demonstrated in early undifferentiated cultures of primary calvarial osteoblasts, with levels becoming undetectable as differentiation progresses (Dodig *et al*, 1996; Dodig *et al*, 1999). Moreover, overexpression of *Msx2* has been found to inhibit differentiation, down-regulate most markers of osteoblastic differentiation, and increase alkaline phosphatase expression in chick calvarial osteoblasts (Dodig *et al*, 1996; Satokata *et al*, 2000). The role of MSX2 in the differentiation of osteoprogenitor cells is supported by the evidence that a dominant mutation in the human *MSX2* gene (P148H; results in a substitution for a proline at position seven in the homeodomain) is associated with craniofacial abnormalities (Jabs *et al*, 1993; Ma *et al*, 1996; Winograd *et al*, 1997). In particular, Boston-type craniosynostosis, or precocious fusion of the skull bones develops and in a few supported cases limb deformities result (Jabs *et al*, 1993). In contrast, the phenotype from *Msx2* deficient mice, resembles that associated with the human *MSX2* haploinsufficiency condition in parietal foramina due to defective proliferation of osteoprogenitors at the osteogenic front of calvaria (Satokata *et al*, 2000).

The role of Msx2 in human bone development is further supported by the evidence that *MSX2* has been identified in cDNA from adult human osteoblasts cells and its expression can be stimulated *in vitro* by 1,25-$(OH)_2D_3$ which promotes osteoblastic differentiation (Hodgkinson *et al*, 1993). *Msx2* expression has also been reported in many established cell lines, such as osteoblastic cells (ROS 17/2.8, RCT 3, MB1.8) and pre-osteoblasts (RCT-1) in addition to neonatal rat calvarial cells (Towler *et al*, 1994), but not in non-osteoblastic cells such as ROS 25/1 or undifferentiated MC3T3-E1 cells. *Msx2* expression has been shown to inhibit the expression of osteocalcin and *COL1A1* (Towler *et al*, 1994; Dodig *et al*, 1996).

Msx2 has been implicated in the formation of membranous bones rather than cartilage. In experiments in which mesenchyme from the avian jaw (expresses *Msx2 in vivo*) was cultured with or without epithelium, cartilage formed in either case, but membrane bone formation and the maintenance of *Msx2* expression required the presence of epi-

thelium (see review in Davidson, 1995). In addition, *Msx2* may not be important in the regulation of differentiation of osteoblasts derived from long bone (Dodig *et al*, 1997).

1.7.2 Factors controlling *MSX* gene expression

As yet, very little is known about the molecular pathways in which the Msx genes function. Evidence of regulatory processes upstream of Msx genes is emerging. BMPs, in particular, BMP-2 and -4 have been implicated in the epithelial-mesenchymal tissue interactions with Msx genes. These genes are co-expressed with *Msx1* and *Msx2* at many locations, including rhombomeres, limb buds and tooth germs (e.g., Vanio *et al*, 1993; Watanabe and Ide, 1993; Barlow and Francis-West, 1997; Kim *et al*, 1998). Several other members of the TGF-β family, for example *TGF-β* and *dorsalin*, are also co-expressed with *Msx* genes (reviewed in Davidson, 1995); in addition other growth factors that have been implicated in controlling Msx gene expression, for example exogenous FGF-2 and FGF-4, as well as BMP-4, can induce low levels of *Msx1* in dissociated limb mesenchymal cells *in vitro*.

1.7.3 Downstream targets of Msx proteins

Finally, increasing information is emerging about some of the downstream targets of *Msx1* and *Msx2*. The consensus homeodomain-binding sequence [c/g]TAATTG, has been found in the promoter of the genes encoding the signalling molecule Wnt1 and osteocalcin, as well as in the promoter of *Msx1* itself (suggesting auto-regulation or cross-regulation with *Msx2*). *Msx2*, in combination with MINT, a Msx2 binding protein, have been shown to transcriptionally repress osteocalcin gene expression (Hoffman *et al*, 1994; Ducy *et al*, 1995; Banerjee *et al*, 1996; Ducy *et al*, 1997; Newberry *et al*, 1997; Geoffrey *et al*, 1995; Towler *et al*, 1994; Newberry *et al*, 1999). Furthermore, during tooth development, reciprocal temporo-spatial patterns of *Msx2* and osteocalcin gene expression has been shown (Bidder *et al*, 1998). In addition, the homeodomain transcription factor, Dlx2, induces osteocalcin expression in osteoblasts by antagonising Msx2 transcriptional repression (Zhang *et al*, 1997; Newberry *et al*, 1998).

1.8 Aims

The regulation of genes by growth factors, hormones and transcription factors is important in the development and remodelling of bone throughout life. In particular, the c-Fos proto-oncogene has been implicated in both osteoclast and osteoblast function and the *Msx* homeobox-containing genes have important roles in osteoblast differentiation during development.

A) c-Fos has been shown to be important in regulating the activity and differentiation of osteoclasts and osteoblasts, and shown to be highly expressed in the osteoclasts of patients with Paget's disease, a bone remodelling disease. To understand the role of c-Fos in bone cell function, the aims of this thesis are:
   1) To investigate the role of Fos in osteoclast function, and possibly provide a working model for Paget's disease, this thesis aims to use an *in vivo* gain-of-function approach to generate transgenic mice which overexpress c-Fos in osteoclasts.
   2) To examine the consequence of inducible c-Fos over-expression in an osteoblastic cell line using a tetracycline-repressible gene construct such that the effects of ectopic c-Fos expression on osteoblast proliferation and differentiation may be assessed *in vitro*.
   3) To ascertain the role of ectopic c-Fos expression in osteoblast apoptosis *in vitro*.

B) Previous studies have demonstrated a role for *Msx* genes in bone and tooth development, and the regulation of *Msx2* by the osteotropic hormone, 1,25-$(OH)_2D_3$. To study further the expression of *Msx* genes in osteoblasts, the aims of this thesis are also:
   1) To investigate the possible regulation of *Msx2* by another osteotropic hormone, Parathyroid hormone (PTH), using reporter gene constructs, and analysis of endogenous *MSX* gene expression in osteoblast-like cell lines.
   2) To examine the *in vivo* expression of *Msx1* and *Msx2* in osteoblasts of postnatal mouse bone using *in situ* hybridisation analysis and β-galactosidase expression from *Msx1-lacZ* knock-in transgenic mice.

2. Materials and Methods

2.1 General Abbreviations

α-MEM	α-minimal essential medium + ribonucleosides and deoxyribonucleosides
APS	Ammonium Persulphate
ALP	alkaline phosphatase
ATP	adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pairs
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
cDNA	complementary DNA
CTP	cytidine triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
DEPC	Diethyl pyrocarbonate
1,25-$(OH)_2D_3$	1,α25-Dihydroxy Vitamin D3
DMF	Dimethylformamide
DNase	deoxyribonuclease
DTT	Dithiothreitol
DMEM	Dulbecco's Modified Eagles Medium
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium bromide
FBS	Feotal Bovine Serum
PFA	Paraformaldehyde
G418	Geneticin

GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GTG	genetic technology grade
GTP	guanosine triphosphate
dH_2O	Deionised water
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IMS	Industrial methylated spirits
kb	kilobases
mA	milli amperes
min	minute/s
MOPS	3(N-Morpholino)propanesulphonic acid
mRNA	messenger RNA
NaOAc	Sodium acetate
NEB	New England Biolabs
NTP	nucleotide triphosphate
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
sec	seconds
SDS	Sodium dodecyl sulphate
SSC	standard saline citrate
TAE	tris acetate-EDTA buffer
Tc	Tetracycline
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TE	tris-EDTA buffer
TEMED	N,N,N,N-tetramethylethylenediamine
TESPA	3-aminopropyltriethoxysilane
TRAP	Tartrate Resistant Acid Phosphatase
Tris	Tris(hydroxymethyl)aminomethane

tRNA	transfer ribonucleic acid
TTP	thymidine triphosphate
Tween 20	Polyoxyethylene sorbitan monolaurate
UTP	uridine triphosphate
UV	ultraviolet
V	Volts
v/v	volume/volume
w/v	weight/volume
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-galactosidase

2.2 Standard Solutions and Media

All chemicals were obtained from BDH (Poole, UK), biochemicals were obtained from Sigma (Poole, UK) and all enzymes were from Promega (Southampton, UK) unless otherwise stated. All cell culture media and supplements were cell culture grade obtained form Gibco BRL (Paisley, UK) or Sigma. Batch tested fetal calf serum for tissue culture use was obtained from Meldrum (Hants, UK), Gibco BRL and Summit (Denver, CO, USA).

2.3 Manipulation of DNA

2.3.1 Ethanol precipitation of DNA

DNA was routinely precipitated by the addition of 0.1 volumes of 3M NaOAc followed by 2.5 volumes of ethanol, and precipitating the mixture at -80°C for 1 hour. The precipitate was isolated by centrifugation for 15 min at the maximum speed possible (usually 14,000 rpm) for the tube used. The pellet was washed with 70% ethanol before suspending in TE (10mM Tris-HCl, 1mM EDTA) or dH_2O.

2.3.2 Plasmid transformation of *E.coli*

2.3.2.1 Preparation of chemo-competent *E.coli*

XL1-B or DH5α *E.coli* cells from a frozen stock were streaked out onto LB agar plates (1.5% Bacto agar [Oxoid] in LB medium) containing 15μg/ml of tetracycline. A single colony was used to inoculate 5ml of LB medium (1% w/v bactotryptone [Oxoid], 0.5% w/v yeast extract [Oxoid], 0.17M NaCl, pH7.5) supplemented with 15μg/ml of tetracycline. The culture was incubated overnight at 37°C with shaking at 250 rpm in a New Brunswick G25 orbital incubator. A volume of overnight culture (1ml) was transferred to a sterile conical flask containing 100ml of LB medium and grown until a 600nm absorbance of 0.94-0.95 was achieved. The cells were then transferred to two 50ml polypropylene centrifuge tubes (Beckman) and chilled on ice for 15 min. The cells were pelleted by centrifugation at 2500 rpm at 4°C for 15 min in a Beckman JA-20 rotor. The supernatant was discarded and the pellet was resuspended in 10ml of Comp. Buffer 1 (80mM calcium chloride, 50mM magnesium chloride) and left on ice for 10 min. Pelleting and resuspending was repeated twice, the second time the pellet being resuspended in Comp. Buffer 2 (100mM calcium chloride) at $5x10^9$ cells/ml. An equal volume of glycerol was slowly added and the mixture was aliquoted into 1.5ml eppendorf tubes. This was frozen in liquid nitrogen and stored at -80°C.

2.3.2.2 Transformation of chemo-competent *E.coli*

Frozen aliquots of competent cells were thawed on ice. DNA solution (approximately 1ng) was added to cells (100μl) in pre-chilled 1.5ml eppendorf tubes. The tubes were incubated on ice for 30 min after which time the cells were heat shocked at 42°C for 90 sec, and placed on ice for further 2 min before adding 500μl SOC medium (2% w/v bactotryptone, 0.5% w/v yeast extract, 5.5mM NaCl, 2.5mM KCl, pH7, 10mM $MgCl_2$). The transformed cells were then incubated for 30 min shaking at 37°C after which time cells (50μl, 100μl, 200μl aliquots) were plated onto LB agar plates containing 100μg/ml ampicillin and incubated overnight at 37°C.

2.3.3 Small scale preparation of plasmid DNA (mini-prep)

Individual colonies were inoculated into 3ml of LB medium containing 100µg/ml ampicillin and incubated overnight at 37°C in loosely capped 15ml tubes shaking at 250rpm. Aliquots of 1.5ml were transferred to eppendorf tubes and pelleted at 2,000g for 10 min. Meanwhile, the remaining culture was stored at 4°C. Plasmid DNA was extracted from bacterial cells using a Qiagen plasmid extraction kit (Qiagen, Crawly, UK) according to manufacturer's instructions.

2.3.4 Large-scale preparation of DNA (maxi-prep)

The 100µl aliquot of cell suspension saved from the mini-preparation (see above) was added to 250ml of LB medium containing 100µg/ml ampicillin in a sterile conical flask and incubated overnight at 37°C with shaking at 250 rpm. Plasmid DNA was isolated using a Qiagen Plasmid Amplification Kit (QIAfilter Plasmid Midi/Maxi protocol) according to manufacturer's instructions. The concentration of DNA in solution was determined by optical density (OD) at 260nm and 280nm using a Pharmacia GeneQuant I spectrophotometer.

2.3.5 DNA cloning

Specific DNA fragments were purified from a plasmid backbone or any unnecessary DNA by restriction digestion. For the restriction digestion of DNA, 2µl of DNA from the small-scale preparation of plasmid DNA was mixed with 2µl of the appropriate 10x restriction enzyme digestion buffer (Promega or NEB), 1 unit of the appropriate enzyme (Promega or NEB), and dH_20 was added to a final volume of 20µl. The mixture was incubated for 1 hour at 37°C in an eppendorf tube. When larger quantities of DNA were required such as in preparation of DNA for production of DNA probes, the final volume and constituents were suitably scaled up.

DNA was resolved by gel electrophoresis on GTG agarose gels containing 0.5µg/ml EtBr in 1x TAE buffer (40mM Tris-HCl, 5mM NaOAc, 1mM EDTA, pH7.7) on a strength of agarose (ICN) appropriate for the separation (usually between 0.6 – 1.2%). The DNA samples were mixed with gel-loading buffer (6x concentrated solution: 15% Ficoll [Type 400; Pharmacia], 0.1% bromophenol blue, 0.1% xylene cyanol

FF, 0.1% Orange G) and loaded onto the gel alongside 5µl of a molecular weight size marker (Promega). When the correct sized band was sufficiently distinct (against a suitable size DNA marker) it was excised from the agarose gel slice under UV illumination and purified with QIAquick gel extraction kit or Qiaex gel isolation resin (Qiagen) according to the manufacturer's instructions.

2.3.5.1 Digestion of DNA for microinjection and preparation of probes

TRAP-*c-fos*LTR construct

The murine TRAP promoter (pBS-TRAP; obtained from Dr. G.D. Roodman, San Antonio, see Boyce *et al,* 1995) was previously fused to full length genomic sequence of the murine *c-fos* gene in which the 3' destabilising sequences have been replaced by a 3' long terminal repeat (LTR) from the FBJ-murine sarcoma virus (TRAP-*c-fos*LTR). The construct (6.9kb) was removed from the plasmid pBluescriptSK using HindIII restriction sites for the microinjection into fertilised eggs. A 0.8kb BamHI v-*fos* fragment, isolated from the pXfos vector, was used as a DNA probe for the Southern blot analysis to detect 1.8kb exogenous c-*fos* DNA (see also Chapter 3, Figure 1).

DNA probes

Probes were prepared by restriction enzyme digestion of the following plasmids to detect specific transcripts in Northern blot analysis:

Probe	Size (kb)	Restriction Enzyme/s	Plasmid	Transcript size (kb)
v-fos	0.8	BamHI	pXfos	*
fra-1	0.23	EcoRI, XbaI	pfra-1-8	4.2
c-jun	0.45	EcoRI, XbaI	pc-jun EXho2	0.45
MMP-9	0.3	EcoRI, SmaI	pSP64	2.3
Msx2	0.3	HindIII, PstI	pBsSK	1.3
GAPDH	1.0	BamHI	pG3PCR-4	1.4
ALP	2.5	EcoRI	pAT153	2.5

* = *v-fos* probe: Detects 2.2kb endogenous and 2kb and 3.0kb exogenous c-*fos* transcripts in TRAP-*c-fos*LTR mice and 1.8kb exogenous transcript from pJMF2-c-*fos* construct.
pBsSK = pBluescript SK

Riboprobes

Plasmids was linearised with the following enzymes for specific anti-sense and sense riboprobes; the table also shows the polymerase enzymes and hydrolysis times used for *in vitro* transcription for riboprobe synthesis:

Probe		Restriction enzyme	Polymerase enzyme	Size (kb)	Plasmid	Hydrolysis time
pB15	sense	EcoRI	T3	0.49	pBsSK	11 min 48 sec
	anti-sense	SalI	T7	0.49	pBsSK	11 min 48 sec
MMP-9	sense	EcoRI	SP6	0.3	pSP64	-
	anti-sense	BamHI	SP6	0.3	pSP65	-
Msx1	sense	EcoRI	T7	0.7	pSP72	17 min 20 sec
	anti-sense	BglII	SP6	0.7	pSP72	17 min 20 sec
Msx2	sense	BglII	SP6	0.85	pSP72	24 min 27 sec
	anti-sense	HindIII	T7	0.85	pSP72	24 min 27 sec

pBsSK = pBluescript SK

2.3.6 Preparation of a Random Prime DNA Probe

The cDNA sequence to be used as a template for making [α-^{32}P]-dCTP-labelled probes was isolated from a plasmid by restriction endonuclease digestion followed by agarose gel isolation and purification (see section 2.3.5). The DNA template (25ng) was heat denatured at 95-100°C for 2 min before cooling on ice. The DNA was random prime labelled using either a Prime-a-Gene™ System (Promega) or Ready-To-Go™ DNA labelling beads (Amersham-Pharmacia Biotech, Amersham, UK) according to manufacturer's instructions, and [α-^{32}P]-dCTP (50μCi, 3000Ci/mmol; NEN, Boston, MA) was used as a choice of isotope labelled nucleotide.

The probe was purified from unincorporated nucleotides using ProbeQuant™ G-50 Micro columns, (Amersham Pharmacia Biotech) according to the manufacturers instructions. The probe was denatured by heating to 100°C for 2 min or by the addition of NaOH (0.1N final concentration), before addition to Church's Buffer (0.5M Na_2HPO_4 pH7.2, 7% SDS, 1mM EDTA, pH8.0) in the hybridisation bottle (section 2.7.3 and 2.8.4).

2.4 Production of Transgenic Mice

Mice were generated using standard transgenic techniques by the department's transgenic facilities and a dedicated technician for these procedures. All procedures were carried out under Home Office license.

2.4.1 TRAP-c-*fos*LTR transgenic mice

For the generation of TRAP-c-*fos*LTR transgenic mice, a HindIII fragment of TRAP-c-*fos*LTR construct (section 2.3.5.1) was microinjected into fertilised eggs according to standard procedures, followed by transfer into foster females. Founder animals were screened by Southern Blot analysis of tail DNA for the presence of the transgene (see section 2.7). X-rays were taken of anaesthetised mice at different ages (Dr. P. Liepins, Dept. of Radiological Sciences, Guy's Hospital, London).

2.4.2 *Msx1-lacZ* transgenic mice

Wild type ($^{+/+}$) female mice (B6CBA strain) were mated with *Msx1-lacZ* heterozygous ($^{+/-}$) males (obtained from Benoit Robert, Institut Pasteur, France). These heterozygotes have the *nlacZ* reporter gene inserted into the *Msx1* homeobox domain resulting in a null mutation but show normal development (Houzelstein *et al*, 1997). Expression of *Msx1* gene can be monitored in heterozygous offspring by β-galactosidase histochemistry. Identification of heterozygotes was assessed by detecting β-galactosidase activity in hemi-sected heads (embryonic age days 16.5 and 18.5 and neo-natal; Day 0 being the sign of vaginal plug after mating) or ear tissue removed from offspring after post-natal age day 6 (section 2.5.6)

2.5 Histochemistry

2.5.1 Harvesting of Mouse Embryos and offspring

For control tissue, mice of CD1 strain (Charles River, Margate, UK), were mated overnight. Embryos at specific stages were dissected from euthanised mothers and extra-embryonic membranes. Limbs and calvaria were removed with as little surrounding muscle as possible, and fixed in 4% PFA (w/v in PBS). Post-natal CD1 mice and TRAP-c-*fos*LTR mice were culled at the indicated stages of development and tissue

transferred immediately to 4% PFA. For embryos and early post-natal tissue the tissue was fixed overnight at room temperature and 48 hours for later staged mice. Bone tissue of mice older than 2 days were decalcified with 0.5M EDTA, until bone could be easily cut with a scalpel blade. The PFA was removed and the embryos were washed successively for 10 min with depc-PBS before paraffin vacuum infiltration embedding whereby tissues were dehydrated through graded ethanol series and toluene and infiltrated with paraffin at 58°C overnight under vacuum (by the Department of Oral Pathology, Guy's Hospital, London).

2.5.2 Sectioning of paraffin embedded tissue

Paraffin embedded samples were sectioned on a Reichert-Jung 1140/Autocut microtome (Lamb) using disposable microtome blades (Lamb) to produce 5µm thick sections. Sections were floated onto water in a water bath containing depc-H_2O pre-warmed to 45°C. As the wax melted, sections were mounted onto organosiliconised (TESPA) slides, left to air-dry at 37°C for 2 hours, before incubating overnight at 50°C. Sections were stored at 4°C until ready for use.

2.5.3 Histological staining

Unless otherwise stated sections were stained with Haematoxylin and Eosin or Eosin alone. Sections were dewaxed in Histoclear for 10 min at room temperature and then passed through a graded series of ethanols (100%, 90%, 80%, 70%, 50%, 30% (v/v) for 2 min, followed by dH_2O. Slides were stained in water-based Haematoxylin for 1 min and rinsed in dH_2O for 2 min. Sections were destained for 1 min in acid/dH_2O (approximately 2 drops HCl in 400ml dH_2O), before rinsing in dH_2O for 2 min. Sections were dehydrated in 30%, 50% and then 70% ethanol before staining for 30 sec in Eosin (Eosin Yellowish in 80% ethanol; Sigma). Finally, sections were dehydrated further in 90% (2 min), 95% (1 min) and 100% ethanol (twice for 30 sec). Sections were cleared in DPX mounting medium (BDH) and covered with glass coverslips (Chance, Proper, England).

2.5.4 Immunohistochemistry

The immunolocalisation of c-Fos and Bcl-2 proteins in sections from TRAP-c-*fos*LTR mice was assessed. All steps were performed at room temperature unless otherwise stated. Sections were dewaxed in Histoclear (twice for 20 min) and rehydrated as above with the exception of a final 5 min wash in Tris buffered saline (TBS; 10mM Tris-HCl pH8.0, 150mM NaCl), instead of dH_2O. For samples to be assessed for Bcl-2 protein, sections were warmed in 0.2M Tris pH7.8 at 37°C for 5 min, before antigen retrieval in 1% trypsin in 0.2M Tris pH7.8 (w/v) at 37°C for 10-30 min. Trypsin was washed away by immersion in TBS for 5 min. The endogenous peroxidase activity for all samples was then blocked by rinsing in methanol containing 3% hydrogen peroxide solution (v/v) for 30 min at room temperature. Sections were rehydrated (2 min for each step) in 90%, 80%, 70% and 50%, 30%, 0% (v/v) IMS/dH_2O then washed in dH_2O (5 min). Permeabilisation was carried out in 1% SDS in TBS (w/v) for 5 min, followed by rinsing in TBS (twice for 5 min). Any non-specific binding sites were blocked by incubation in 10% Goat serum (v/v) and 2% BSA (w/v) in TBS for 30 min, before primary c-Fos and Bcl-2 antibodies (both were batch-tested rabbit polyclonal IgGs, obtained from Santa Cruz laboratories, CA, USA) were applied at a dilution of 1:100 in 10% Goat serum v/v in TBS to each section and incubated for 1 hour at room temperature. Negative controls consisted of TBS alone. Sections were then washed three times in TBS before being incubated for 1 hour with a 1:300 dilution of the secondary antibody (biotinylated Goat-α-rabbit, Vector Laboratories, Burlingame, CA, USA) in TBST (0.05% Tween 20 in TBS, pH8.0). The antibody was washed away by three washes using TBST and the sections were stained for 30 min with Avidine Biotin Complex (Vector laboratories). Any excess stain was removed by washing in TBST. Sections were permeabilised in 0.5% triton in TBS (v/v) for 5 min before staining with Sigma Fast DAB (Diaminobenzidine; Sigma) for approximately 10 min. The excess stain was removed with dH_2O before the slides were counterstained for 4 sec with Haematoxylin and destained briefly in dH_2O. Sections were mounted in Aquamount (BDH, Merck).

2.5.5 TRAP Histochemistry

For the identification of TRAP positive osteoclasts, bone sections were dewaxed and rehydrated to dH_2O as described above and then into PBS, before incubation at 37°C in TRAP staining solution. This was prepared by first dissolving Naphthol-AS-TR-phosphate (60mg) in 300µl dimethylformamide (DMF). Acetate buffer containing 20mM Glacial acetic acid and 80mM anhydrous sodium acetate, (pH5.2), was added to a final volume of 60ml. Sodium tartrate (1.38g) was added to a final concentration of 100mM. Whilst this was mixed, 60mg Fast Red TR salt was added, and the final solution filtered through a 0.2µm filter unit before the pH was confirmed to be pH5.2. Following the appearance of TRAP positive cells (approximately 20 min), sections were washed with dH_2O, before being counterstained briefly with Haematoxylin as described above, and mounted in Aquamount.

2.5.6 Staining of foetal tissue and bone for β-galactosidase activity

For the identification of heterozygous embryos of *Msx1-lacZ* mice, embryos were dissected from euthanised mothers and extraembryonic membranes, and placed in glass dishes. Half of the hemi-sected head and limbs of each embryo were fixed overnight in 2% formaldehyde, 0.2% glutaraldehyde, 0.02% Nonident P-40, and 0.01% Sodium deoxycholate in PBS. These were washed for 1 hour in PBS and then incubated in X-gal staining solution (10mM potassium ferricyanide, 10mM potassium ferrocyanide, 2mM $MgCl_2$, and 20mM X-gal in PBS) at 37°C until a blue colour developed (about 24 hours). Similarly the ear tissue of post-natal mice were fixed for 1 hour and stained with X-gal.

The remaining halves of limbs and heads from embryonic and post-natal mice were used for analysis of *Msx1-lacZ* expression. However a novel procedure was used to prepare bone samples which helped to eliminate the unnecessary background staining that can be observed with neonatal samples of bone or bone which has been mineralised (recommended by Diana Cummings, University of Maryland, USA). Freshly, dissected bone tissue was fixed in 4% PFA in PBS overnight. Foetal samples were divided such that half limbs or hemi-sected head of the animal were stained prior to demineralisation and the other half after demineralisation and sectioning to confirm that demineralisation

did not inhibit β-galactosidase activity. Tissue was demineralised in 25% Formic acid/7.5% sodium citrate at 4°C for 6 hours and cryoprotected in 30% sucrose (w/v in PBS) overnight before freezing by quick immersion in a methanol/dry ice bath and storing at -80°C. Sections (5-7μm) were prepared using a cryostat at -40°C and adhered to polylysine coated slides (BDH). Sections were fixed in 4% PFA in PBS and stained for β-galactosidase activity, as above, and counterstained with Safranin (0.01% w/v in dH_20). Adjacent sections were stained with Haematoxylin and Eosin as described above. Due to the problem of background staining of β-galactosidase, ear and bone tissue from wild-type mice was used as a negative control. Also, sections from late stage embryonic (stage E17) to early post-natal (day 3) tissue were included as a positive control for expression analysis (see Chapter 6.5).

2.6 Tissue Culture

2.6.1 Mammalian Cell lines and stock solutions

The following cell lines were used in these studies: the human osteosarcoma cell lines SaOS-2 and MG63 (both from American Type Culture Collection, Manassas, VA, USA), the rat osteosarcoma cell line ROS 17/2.8 (gift from Professor Gideon Rodan, Merck Research Laboratories, Merck & Company Inc, West Point, PA, USA), murine MC3T3-E1 osteoblastic cells and Monkey COS cells (both from European Collection of Cell Cultures, Salisbury, Wiltshire, UK). Also, primary rat osteoblastic cells, supplied by D. Harmey, were obtained by sequential enzymatic digestion of 21-day fetal rat calvaria using a modification of the methods of Bellows *et al* (1998).

The following stock solutions were prepared in tissue culture grade dH_2O and stored at -20°C, unless otherwise stated:

Tetracycline	1mg/ml stock in dH_2O, filtered (using 0.2μm filter unit)
Ascorbic acid	5mg/ml stock in dH_2O, filtered (using 0.2μm filter unit)
β-glycerophosphate	1M stock in dH_2O, filtered (using 0.2μm filter unit)
BMP-2/BMP-4	10-200μg/ml in 0.2%BSA in PBS, stored at -80°C (Genetics Institute, Cambridge, MA)
PTH	Amino acid fragment 1-34; 100mM stocks in PBS

$1,25\text{-}(OH)_2D_3$	10mM stock in ethanol (100%) from Calbiochem (Nottingham, U.K.)
Dexamethasone	100mM stock in ethanol (100%)

2.6.2 Maintenance of osteoblast-like cell lines

All cells were manipulated under sterile conditions in a Class II cabinet. Cells were maintained at 37°C in an atmosphere of 5% (v/v) CO_2 in air (Forma Scientific). SaOS-2, ROS 17/2.8 and MG63 cells were maintained in DMEM (Sigma) supplemented with 10% heat-inactivated FBS (E.C. approved from Gibco BRL), 1% glutamine (200mM L-glutamine and 1% penicillin and streptomycin (5,000 units/ml penicillin, 5,000mg/ml streptomycin) (Gibco BRL). MC3T3-E1 cells and primary rat osteoblasts were similarly maintained, but with the exception of α-MEM instead of DMEM. FBS from Meldrum or Gibco BRL was used for medium for most experiments except for differentiation experiments when FBS from Summit was used. Cells were sub-cultured when monolayers were confluent in a 1:5 ratio approximately twice weekly, by treatment with 0.05% (w/v) trypsin-EDTA at 37°C for 5 min to ensure release from the culture vessel and the trypsin was then neutralised with culture medium.

2.6.3 Estimation of cell concentration

To count the cells for experiments, cells in culture were trypsinised as above and suspended into a suitable volume of medium. A sample of cell suspension was diluted 1:10 with 0.4% (w/v) trypan blue (Sigma) and counted in a haemocytometer (the trypan blue is excluded from viable cells). The cell suspension was the diluted into the required concentration.

2.6.4 Cryopreservation of cells

Following treatment with trypsin, cell pellets were resuspended in 40% FBS, 50% culture medium and 10% DMSO at approximately $1x10^6$ cells/ml and frozen at -80°C overnight in cryotubes (Nunc, Gibco) in an insulated container and then transferred to a liquid nitrogen cell storage bank.

2.6.5 Stable transfection of MC3T3-E1 with pJMF-2-c-*fos* and pJMF-2-*luc* vectors

Exponentially growing MC3T3-E1 cells were transfected using Effectene reagent (Qiagen) according to manufacturer's instructions. Cells were seeded at 10^6 cells per 10cm dish and incubated overnight. Transfection with pJMF-2-c-*fos*, pJMF-2-*luc* vectors or minus DNA control was carried out overnight in the presence of Tc (10μg/ml), according to Qiagen's recommendations for 10cm dish.

Following 48 hours in culture the transfected cells were passaged from each dish into 4 dishes at varying densities. Cells were cultured with 300μg/ml G418 (Sigma) for two weeks to allow for colony selection. Medium was replaced every 2-3 days. Cell death was abundant and individual cells gave rise to well separated colonies. Control (no DNA) transfections resulted in complete cell death within 4 days. Individual clones were picked by first washing the culture vessel with PBS, followed by isolating the area using steel cloning rings which had been carefully coated with silicone gel around the base. Colonies were treated by gentle trypsinisation to release the colonies and transferred to the wells of a 48-well plate, for clonal propagation. Clones were propagated in culture for analysis of genotype including exogenous c-Fos expression (KT1.1-22; section 2.8.4) and luciferase activity (KT2.1-7; section 2.6.6.4).

2.6.6 Transient transfection of cells

2.6.6.1 Transfection of p21 and Bcl-2 in KT1.5 cells using Effectene

To assess the affects of the CDK2 inhibitor p21 and anti-apoptotic protein Bcl-2 on c-Fos-induced apoptosis, KT1.5 cells were transfected with p21 or Bcl-2 expression plasmids. KT1.5 cells were cultured on glass coverslips in 6-well plates in the presence and absence of Tc for 24 hours to induce c-Fos expression. Three wells were allocated per group. Cells were transfected overnight in the absence of Tc with pcDNA-3, p21-HA (Dr. A. Yeudall, Craniofacial Development Dept., King's College London) and pBcl-2 vectors (Dr. S. Korsmeyer, Depts of Pathology and Medicine, Harvard Medical School, Boston, MA) or in the absence of DNA using Effectene reagent following manufacturer's instructions for 6-well plate. In addition, a secreted placental alkaline

phosphatase (SPAP) reporter vector (pCMV-SPAP; a gift from Glaxo SmithKline, Stevenage, UK), was included as the internal control for transfection efficiency between groups. This reporter product codes for a heat resistant secreted placental alkaline phosphatase, which is secreted into the medium and can be easily detected without disturbing the cells. Following the removal of transfection medium, cells were cultured for a further 24 hours with standard medium ± Tc. Culture medium was assayed 24 hours later for SPAP activity (detailed in section 2.6.6.3), with three samples per transfection group. Cells were washed three times with PBS before apoptosis was induced by serum withdrawal (0% FCS, 0.1% BSA), ± Tc for 24 hours. Cells were then fixed and stained with propidium iodine for assessment of apoptosis (section 2.6.11).

2.6.6.2 Lipofection of cells with p*Msx2*-CAT, pSV40-CAT and pCAT-Basic vectors

Factors known to be important in bone development, in particular PTH, were screened for their ability to induce the expression of two murine *Msx2*-CAT (Chloramphenicol acetyltransferase) reporter gene constructs containing the 1.2kb or 2kb *Msx2* upstream regulatory sequence. The 1.2kb *Msx2*-CAT construct (Figure 8.1 -Appendix) was prepared by Jane Hodgkinson (PhD thesis, 2000) by inserting a murine 1.2kb *Msx2* promoter fragment into pCAT-Basic (Promega) upstream of the CAT coding sequence in this plasmid. The 2kb *Msx2*-CAT construct was a gift from Dr. R. Maxson, Los Angeles, USA (Liu *et al*, 1994; Liu *et al* 1999). The 2kb *Msx2* fragment used in this construct is located approximately 3kb upstream of the transcription start site (*pers. comm*).

Furthermore, the control plasmids that were used included pCAT-basic (Promega), a negative control for transfection, and pSV40-CAT (Promega), which consists of pCAT-basic and SV40 promoter sequence, a strong constitutive promoter and hence positive control. In addition, pCMV-SPAP was included as the internal control for transfection.

Since the efficiency of lipofection is dependent upon many factors, preliminary experiments were designed to establish suitable quantities of Lipofectamine™ (Gibco BRL) and DNA according to manufacturer's instructions. From this experiment the

highest lipofection efficiency was observed in samples when 1μg DNA and 8 μl Lipofectamine were used in 1ml of culture medium (data not shown).

Cells were seeded into 6-well plates 24 hours before transfection so that the cells were approximately 60% confluent for the time of transfection. The lipofection of cells was carried out using 8μl/ml Lipofectamine and 1μg/ml of the following vector DNAs: 1.2kb *Msx2*-CAT, 2kb Msx2-CAT, pCAT-basic, or pSV40-CAT according to manufacturer's instructions for 6-well plates. pCMV-SPAP (0.2μg/ml) was also included in each of the aforementioned transfection reactions as an internal control for transfection efficiency. Cells were transfected overnight in serum-free conditions, before medium was changed to 10% FCS by the addition of an equal volume (1ml) of 20% culture medium for a further 5 hours. By this time, cells had recovered, were passaged by trypsinisation into the wells of a 6-well plate, and cultured for a further 24 hours before a sample of medium was taken for assessment of SPAP activity (section 2.6.6.3). Cells were treated with osteotropic factors or vehicle control in 2% foetal bovine serum for up to 24 hours. These factors included PTH, 1,25-$(OH)_2D_3$, BMP-2, BMP-4, FGF-4, FGF-8, Sonic hedgehog and dexamethasone concentrations as indicated.

Following treatment, cell extracts were prepared in Promega's 1x Reporter Lysis Buffer (300μl) and CAT activity monitored by assessing the incorporation of ^{14}C-chloramphenicol (ICN Flow) using liquid scintillation counting according to the Promega protocol. CAT activity was represented in counts per minute (CPM). Samples were standardised for protein content (section 2.9.4) and SPAP activity to control for transfection efficiency. Due to the fact that there was variability in the levels of CAT activity (or CPM) between experiments the data was standardised between experiments by expressing CPM for treatment as a ratio of the CPM for the control (T/0: the relative CAT activity).

Co-transfecting cells with constructs, such as CMV-SPAP, can result in a lower transfection efficiency and reporter gene response. Therefore, a comparison was made between *Msx2*-CAT-transfected and CMV-SPAP/*Msx2*-CAT co-transfected SaOS-2 cells in their response to PTH. SaOS-2 cells were transfected with p*Msx2*-CAT (1.2kb construct) alone or p*Msx2*-CAT plus pCMV-SPAP vector. Following transfection,

cells were treated for 24 hours with PTH (10nM), before extracts were prepared for assessment of CAT activity as described above.

2.6.6.3 Estimation of SPAP activity

Alkaline phosphatase cleaves the phosphate group from the substrate *p*-nitrophenyl phosphate (pNPP) yielding a yellow-coloured product, *p*-nitrophenol (pNP), whose absorbance can be measured at 405nM. Samples of medium were taken at 24 hours to confirm transfection efficiency by measuring the amount of SPAP activity before any treatment was added (Tate *et al*, 1990). To do this, 50µl spent medium was heat-inactivated at 65°C for 30 min, to inactivate any endogenous alkaline phosphatase. PNPP reaction mixture (1ml) was added, mixed and transferred to a plastic cuvette, then the reaction was developed in the dark. This reaction mixture consisted of 5mM p-nitrophenylphosphate (PNPP) in DEA buffer (1M diethanolamine, 0.28M NaCl, 0.5mM $MgCl_2H_2O$, pH9.85). The absorbance at 405nm was measured when a yellow colour started to develop. The concentration of SPAP in the original medium in units/ml were calculated as follows:

$$[\text{SPAP}]\ \text{units/ml} = \text{Abs}_{405\text{nm}}/t \times 18.5 \times \text{Vol}$$

where, $\text{Abs}_{405\text{nm}}$ = Absorbance at 405nm,

t = time of PNPP reaction (in min),

Vol = volume of medium assayed (ml).

2.6.6.4 Estimation of Luciferase activity in KT luciferase clones

KT luciferase clones (KT2.1-2.7) and AT7.8 (positive control) were plated in a 6-well plate and cultured for 4 days ± Tc (10µg/ml). Cell lysates were prepared in 1x Reporter Lysis Buffer (Promega; 145µl/well). Luciferase activity (20µl cell extract) was assessed according to manufacturer's instructions, using a luminometer (10 sec reading) and the amount of light intensity produced was standardised for the protein content of each sample (section 2.9.4).

2.6.7 Osteoblast differentiation

Wild type (wt) MC3T3-E1 cells, c-Fos clones KT1.5 cells and AT9.2 cells, and the Luciferase clone KT2.1 were cultured to induce differentiation for a minimum of 30

days. Cells were plated at a density of $2x10^4$ cells/well of 24-well plate, in standard medium: α-MEM supplemented with 10% FCS (Meldrum) and Tc (10μg/ml). The culture medium was replaced after 24 hours with differentiation medium: α-MEM supplemented with 10% FCS (Summit), 50μg/ml ascorbic acid, 10mM β-glycerophosphate, Tc (0, 1μg/ml) and BMP-2 (0, 100ng/ml). The medium was replaced every 3 days. After 30 days, cultures were fixed in 4% formaldehyde in PBS and stained for alkaline phosphatase activity and mineral as described in section 2.6.8. In addition, similar cultures were set up for mRNA expression analysis by RT-PCR (sections 2.8.5) and quantitative assessment of alkaline phosphatase activity as described in 2.6.9.

2.6.8 Histological identification of alkaline phosphatase

Cells were fixed as previously described and the cells were rinsed with dH_2O, and then incubated in dH_2O for 15 min before the substrate for alkaline phosphatase was added. For 50ml substrate 5mg Naphthol AS MX-PO_4 was dissolved in 200μl DMF. This was mixed with 25 ml 0.2M Tris-HCl pH8.3, 25 ml dH_2O and 30mg Red Violet or Fast Red TR Salt. The mixture was filtered (0.2μm filter unit) immediately before it was added to the cell layer. Cells were incubated with substrate for approximately 20-30 min at 37°C, before the stain was washed off with three changes of dH_2O and finally stored in dH_2O.

2.6.9 Quantitative assessment of alkaline phosphatase activity

KT1.5 and KT2.1 cells were cultured in differentiation medium including Tc (0, 1μg/ml) and BMP-2 (0, 100ng/ml) for 35 days as described in section 2.6.7. Cells were washed with PBS and scraped from the well into an eppendorf tube with 200μl 50mM Tris-HCl, pH7.4. Cells were sonicated (20 sec on ice) and pelleted by centrifugation at 5000 rpm for 30 min at 4°C. The supernatant was tested for alkaline phosphatase (ALP) activity as described section 2.6.6.3 with the exception that extract was not heat-inactivated before assessment. Protein concentration per sample was determined using the BCA protein assay reagent (section 2.9.4) so that alkaline phosphatase activity could be expressed in units ALP/μg protein/well.

2.6.10 Osteoblast proliferation assay

For analysis of proliferation, MC3T3-E1, KT1.5 and KT2.1 cells were plated (in 24- and 48-well plates at between 10^2 and 10^4 cells/cm^2) in standard medium (as above) for 24 hours, then changed to differentiation medium containing Tc and BMP-2, or remained in standard medium, as indicated for each experiment. Cells from triplicate wells were individually trypsinised from the culture vessel and cell numbers counted (section 2.6.3) at the indicated times.

2.6.11 Assessment of osteoblast apoptosis by Propidium Iodine and TUNEL

Cells were stained with propidium iodine (PI) for the assessment of apoptosis in all experiments. Cells grown on glass coverslips (12mm diameter) were fixed for 5 min with 50% acetone in methanol (v/v) pre-chilled to -20°C. Coverslips were air-dried briefly (2-5 min), before cells were rehydrated with PBS. Coverslips were stored at 4°C until analysis. Excess PBS was removed before cells were stained with 100µg/ml PI and 0.1mg/ml RNase A in PBS. Coverslips were washed three times with PBS before mounting upside down onto clean glass slides with Vectashield mounting medium (Vector Laboratories) for analysis using fluorescence microscopy. Apoptotic nuclei were quantified by counting at least 100 cells per field in 5 fields of view per coverslip.

DNA strand breaks during apoptosis were determined by TUNEL (TdT-mediated dUTP nick end labelling) assay using the In Situ Cell Death Detection, POD kit (Roche, Germany). In this histochemical test, the Terminal deoxyribonucleotidyl transferase (TdT) enzyme attaches fluorescein-labelled nucleotides (dUTP) to the 3'-OH group of broken DNA. Incorporated fluorescein is detected by anti-fluorescein antibody Fab fragments from sheep. In one experiment, cells grown on glass coverslips were fixed with PFA for 1 hr at room temperature and rinsed in PBS. The cells were permeabilised (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Coverslips were rinsed twice in PBS and excess PBS absorbed away from the coverslip using 3MM paper. Cells were incubated with TUNEL reaction mixture (1:10 dilution of terminal transferase solution in label solution) in a humidified chamber for 60 min at 37°C. Coverslips were rinsed again with PBS (three times), before mounting onto glass slides with Vec-

tashield for analysis using fluorescence microscopy. Negative controls were made by omitting the terminal transferase from the reaction mixture.

2.6.11.1 Assessment of apoptosis in KT1.5 cells induced by serum withdrawal or TNF-α

For the induction of apoptosis, KT1.5 and KT2.1 cells were plated on glass coverslips (4 per well) in a 6-well plate in α-MEM supplemented with 10% FCS and Tc (10μg/ml). After 24 hours the cells were washed three times in PBS and the medium was replaced with medium ± Tc for a further 48 hours. Following three washes with PBS, apoptosis was induced for 48 hours by serum-withdrawal (α-MEM supplemented with 0.1% BSA) ± TNF-α (30ng/ml; R&D Systems) or 10% FCS ± TNF-α before cells were fixed for apoptosis assessment (section 2.6.11).

Caspase inhibitors Z-VAD-fmk and DEVD-CHO (Calbiochem) were used to inhibit the apoptosis induced by serum withdrawal in the presence of c-Fos expression. Preceding induction of apoptosis, cultures were set up as above and then various regimes were used to study these inhibitors:

1) Cells (+ Tc only) were treated complete growth medium (10%) or serum withdrawal (0% FCS, 0.1% BSA), and a range of Z-VAD-fmk concentrations (0, 3, 10, 100μM). After 24 hours treatment cells were fixed and assessed.
2) Cells (± Tc) were pretreated for 24 hours ± Z-VAD-fmk (10μM), before medium was replaced with complete growth medium (10%) or serum withdrawal (0% FCS, 0.1% BSA) ± TNF-α (30ng/ml), and ± Z-VAD-fmk. Cells were treated for further 48 hours before cells were fixed and assessed.
3) Again, cells (± Tc) were pretreated but for 1 or 6 hours ± Z-VAD-fmk (10μM), before apoptosis was induced by serum withdrawal (0% FCS, 0.1% BSA) ± Z-VAD. After 48 hours treatment cells were fixed and assessed.
4) Cells (± Tc) were pretreated for 1 hour ± DEVD-CHO (3, 30μM), before medium was replaced with complete growth medium (10%) or serum withdrawal (0% FCS, 0.1% BSA) ± DEVD-CHO. After 48 hours treatment cells were fixed and assessed.

5) Once more, cells (± Tc) were pretreated for 1 hour ± DEVD-CHO (30μM), before apoptosis was induced by serum withdrawal (0% FCS, 0.1% BSA) plus TNF-α (30ng/ml), ± DEVD-CHO. Cells were treated for further 48 hours before cells were fixed and assessed.

The CDK2 inhibitor, Roscovitine (Calbiochem), was also used to inhibit the apoptosis induced by serum withdrawal in the presence of c-Fos expression. Preceding apoptosis induction, cultures were set up as above, then cells (± Tc) were pretreated for 1 hour Roscovitine (0, 3, 10, 30μM), before apoptosis was induced for 48 hours by serum withdrawal (± Roscovitine) and assessed as before.

The effects of Roscovitine and Z-VAD-fmk were also assessed on Etoposide-induced apoptosis. Cells were set up as above and were pretreated for 1 hour with Roscovitine (30μM) or Z-VAD-fmk (10, 30μM) before induction of apoptosis by Etoposide (100μg/ml; Sigma) for 48 hours.

Finally, cells were quiesced before induction of apoptosis. Cells were cultured ± Tc for 24 hours before quiescence was induced in 0.5% serum for 24 hours, then a further 24 hours ± Roscovitine (30μM in 0.5% serum). Apoptosis was induced for 48 hours by serum withdrawal (0% FCS, 0.1% BSA), or cells remained to be treated with 0.5% serum, ± Roscovitine.

2.6.12 Assessment of cAMP production

The amount of cAMP produced following treatment was assessed in order to confirm the activity of PTH used in the experiments in 2.6.6.2 above. Cells (SaOS-2, MC3T3-E1, ROS 17/2.8) were plated at 1.25 x 10^5 cells per well of 24-well plate and incubated overnight. Cells were first pre-treated with serum-free medium containing 500μM isobutylmethlyxanthine (IBMX; 400μl) or DMSO (1% v/v) for 15 min. IBMX was removed and replaced with serum-free medium containing IBMX (500μM) and PTH (0, 10, 100nm), forskolin (10μM) or DMSO. Following incubation at 37°C for 15 min, the treatment was terminated with the addition of 800μl absolute ethanol at -20°C. The ethanol extract containing the cAMP was removed and transferred to microcentrifuge tubes. The wells of the 24-well plate were washed with 400μl ethanol (66% v/v) and

the extract transferred to the same appropriate tube. Samples were centrifuged in a microcentrifuge and supernatant containing cAMP transferred to a new tube. The ethanol was evaporated using a rotary, pulsed vacuum evaporator for approximately 350 min, at 50°C.

The Biotrak cAMP enzymeimmunoassay (EIA) system (RPN225; Amersham, Pharmacia Biotech) was used to determine the concentration of cAMP, by following the manufacturer's instructions for non-acetylation assay (standards of cAMP in the range of 12.5-3200fmol/well). The reaction was stopped by the addition of sulphuric acid, and the absorbance of reaction measured at 450nm in a microplate reader. Samples and standards were assayed in duplicate. Protein concentration was determined using the Bio-Rad *DC* Protein Assay (as detailed in section 2.9.4) to standardise the samples for cAMP concentration per μg protein (fmol/ μg protein).

2.7 Southern Hybridisation Analysis

2.7.1 Preparation of genomic DNA

For the genotyping of TRAP-c-*fos*LTR mice, DNA was isolated from tail ends by overnight incubation at 55°C in 700μl volumes of 50mM Tris-HCl pH7.5, 100mM NaCl, 10mM EDTA, 1% SDS, 0.5μg/ml proteinase K (10mg/ml stock in depc-dH_2O). The resultant mixture was cleaned by the addition of 500μl of 6M NaCl (a saturated solution) and 5 min continual shaking. The samples were centrifuged at 10,000g for 7 min and 750μl of supernatant removed to a new tube. The DNA was precipitated by the addition of 500μl of isopropanol and mixing for 2 min. The precipitate was then pelleted by 5 min centrifugation at 10,000g and the supernatant removed. The pellet was washed in 800μl of 70% EtOH and the pellet isolated by 5 min centrifugation at full speed and all of the supernatant removed. The pellet was dissolved in 50-200μl TE, left to dissolve overnight at 4°C or incubated at 37°C with gentle shaking for 2 hours.

2.7.2 Southern blotting

For genotyping of TRAP-c-*fos*LTR mice and identification of transgene integration, each DNA sample (10μg) was digested with XbaI restriction enzyme overnight and electrophoresed on 0.8% agarose gel in 1x TAE, and the gel was photographed beside a

ruler. The gel was first depurinated in 0.25N HCl for 20 min with gentle shaking, and then denatured with 0.5M NaOH/1.5M NaCl for 30 min. A sheet of charged nylon membrane (GeneScreen Hybridisation transfer membrane, NEN) and 3MM Whatmann paper were cut to the exact size of the gel and pre-wet in dH_2O for 10 min followed by 10x SSC (dilution of standard 20x stock: 3M NaCl, 300mM sodium citrate, pH7.0). The following Southern transfer was then set up: First, a clean glass plate was laid on top of a sheet of 3MM paper. The gel was inverted and gently placed on top of this, followed by membrane, then 4 sheets of pre-wetted 3MM paper. Air bubbles were eliminated by the rolling of a pipette across the surface after each sequential positioning of the gel, the membrane, and the paper. Saran wrap™ was arranged around the gel so as to prevent a short-circuit of liquid. A 10cm high stack of highly absorbent paper towels, the same dimension of the gel, was placed on top of the transfer blot followed by a weight (approximately 250g). The transfer was allowed to continue overnight. To confirm completion of transfer, the gel was viewed under UV illumination for visualisation of any remaining DNA. The position of the wells was marked in pencil on the membrane, before the membrane was rinsed whilst stationary in 50mM sodium phosphate buffer (pH7.2) for 10 min followed by 10 min shaking. DNA was fixed to the membrane by UV-crosslinking whilst wet at 120V for 100 sec before baking at 80°C for 1 hour. Membrane was then stored between filter paper until hybridisation.

2.7.3 Southern Blot Hybridisation

The membrane was pre-hybridised for 30 min at 65°C with Church's Buffer (0.5M Na_2HPO_4 pH7.2, 7% SDS, 1mM EDTA, pH8.0) in a rolling bottle Techne Hybridisation Oven at all stages. The probe (a 0.8kb BamHI v-*fos* fragment labelled with α-^{32}P as described in section 2.3.6) was denatured for 10 min with NaOH (0.1N final concentration). Fresh Church's Buffer was added to membrane and followed by the denatured v-*fos* probe.

After hybridising overnight, non-specific hybridisation was removed by a series of 15 min washes in Church's wash buffer (1% SDS in 40mM NaPi [89g $Na_2HPO_4 2H_2O$, 3-4ml H_3P0_4, pH7.2]) at 65°C, until removal of background signal was observed. In between each wash, the membrane was temporarily removed from the bottle and moni-

tored using a Geiger counter and the washing stopped once the areas of the membrane, which were not expected to bind probe, were emitting low background readings. The membrane was then wrapped in Saran Wrap and autoradiography performed in an X-ray cassette at -80°C beside a piece of X-ray film (Kodak Scientific Imaging Film, X-OMAT™ AR) and an intensification screen (GRI). If the resultant film revealed high background radioactivity the washing process was repeated.

2.8 RNA extraction, Northern hybridisation analysis and RT-PCR

All stock solutions (e.g., dH_2O, PBS, NaOAc) were treated with 0.01% DEPC overnight and autoclaved at 115°C for 15 min prior to use or where this was not possible (e.g., agarose, SDS, diluted ethanol, NaCl) the constituents were carefully weighed out using RNase-free conditions and suspended in depc-H_2O. All washes and incubations were performed at room temperature with gentle agitation, unless otherwise stated. Following RNA extraction RNA was stores at -80°C.

2.8.1 Extraction of poly $(A)^+$ RNA

Poly $(A)^+$ RNA was extracted by either of the two oligo d(T) column methods: extraction using oligo d(T) cellulose from Roche or by the PolyATtract® Series 9600™ isolation kit (Promega).

Cells were cultured as described in section 2.8.4 and pelleted from the culture vessel by trypsinisation and centrifugation at 1200rpm for 5 min. The pellet was washed once with PBS and centrifuged again, before freezing at -80°C until extraction was performed. The cell pellet was thawed on ice and resuspended in 5ml STE (0.1M NaCl, 20mM Tris pH7.4, 10mM EDTA pH8.0), 200µl proteinase K (10mg/ml) and 250µl SDS (10% w/v stock). Cells were homogenised using a Polytron at setting 8 for 30 sec and incubated at 37°C for 30 min. NaCl (450µl; 5M) and 2ml of 50mg/ml oligo (d)T (Roche) suspended in loading buffer (0.4M NaCl, 20mM Tris pH7.4, 10mM EDTA pH8.0, 0.2% SDS) were then added, and incubated overnight on a slowly moving shaker. Samples were centrifuged at 2,000rpm for 5 min, washed twice with Loading buffer (10ml), and finally resuspended in 10ml Loading buffer to be poured to a Poly-Prep® Chromatography Column (Biorad). This was washed with 1ml Loading buffer,

followed by 10ml Washing buffer (0.1M NaCl, 10mM Tris pH7.4, 1mM EDTA pH8.0, 0.2% SDS). Finally, poly $(A)^+$ RNA was eluted into siliconised 30ml glass Corex tubes with 5ml pre-warmed (37°C) elution buffer (1mM Tris pH7.4, 1mM EDTA pH8.0, 0.2% SDS) and a sample taken for estimation of RNA integrity before being precipitated at -20°C overnight with 30µg transfer RNA (20mg/ml tRNA stock), 0.88ml 2M NaOAc and 12ml absolute ethanol. RNA pellet was produced by centrifugation at 10,000rpm for 45 min at 4°C in Beckman JA-14 rotor, washed and centrifuged again with 70% ethanol, and briefly air-dried before being resuspended at 0.5mg/ml in depc-dH_2O.

Tissue removed from TRAP-c-*fos*LTR mice at autopsy was frozen immediately in liquid N_2 and stored at -80°C until RNA extraction was performed. To extract RNA, tissue fragments were transferred to a 50ml Falcon tube containing 8ml solution D (4M guanidium isothiocyanate, 25mM sodium citrate, 100mM β-mercaptoethanol, 0.5% laurlysarcosine, 0.1% Antifoam) and homogenised using a Polytron at full speed as above (45 sec or longer for bone and calcified tumour samples). NaOAc (800µl; pH4.0), 8ml Tris-saturated phenol, and 1.6ml chloroform were sequentially added with vigorous mixing in between each addition and incubated on ice for 15 min. Samples were transferred to 30ml Corex tubes for phase separation at 10,000rpm for 15 min in Beckman JA-14 rotor. The upper phase was removed and an equal volume isopropanol added for precipitation of RNA (-20°C, overnight). Samples were centrifuged again at 10,000rpm for 15 min to produce an RNA pellet. The pellet was dissolved in 5ml ice-cold STE. Following on from this step, RNA was processed as above for extraction of poly $(A)^+$ RNA from cells.

For the extraction of poly $(A)^+$ RNA by oligo d(T) columns using Promega, PolyATtract® Series 9600™ isolation kit, cells were grown in tissue culture and pelleted from the tissue culture vessel by trypsinisation as before, but the cell pellet was washed with PBS before quick freezing in a methanol/dry-ice bath, until the extraction of poly $(A)^+$ RNA according to Promega's instructions.

2.8.2 Extraction of Total RNA

Cells were pelleted from the wells of 24-well plate by trypsinisation as before. The cells in the pellet were lysed by the addition of 300µl Solution D, followed by the sequential addition of 30µl 2M NaOAc (pH4.0), 300µl phenol (Tris-saturated) and 76µl chloroform, with vigorous mixing in between each addition. The mixture was vortexed for 10 sec and incubated on ice for 15 min. Phase separation was achieved by centrifugation at 10,000rpm for 20 min at 4°C. The aqueous phase was removed and RNA precipitated by the addition of 300µl isopropanol at -20°C overnight. The RNA was recovered by centrifugation at 10,000rpm for 20 min at 4°C, and the pellet was resuspended in 100µl Solution D and 200µl ethanol for a further precipitation overnight at -20°C. Samples were centrifuged at 10,000rpm for 10 min at 4°C, the supernatant was removed and the pellet was washed in 100µl 75% ethanol and centrifuged again. Following removal of the supernatant the pellet was briefly air-dried. The pellet was resuspended in 30µl depc-H_2O.

2.8.3 Northern blotting

All equipment such as gel tank and combs were washed with 0.1M NaOH followed by rinsing in depc-H_2O in order to minimise RNase contamination. The denaturing gel was prepared by dissolving 3.15g high grade agarose (ICN) in 285ml dH_2O and 7ml 50x MOPS (1M MOPS, 0.25M NaOAc, 0.05M EDTA) by boiling. After cooling, 59ml RNase-free formaldehyde was added to the gel mixture.

RNA (10µg) samples were diluted to a volume of 20µl with depc-dH_2O before addition of 20µl formamide, 7µl formaldehyde (40%), 1µl 50x MOPS and 2µl bromophenol blue. Samples were denatured at 70°C for 5 min and immediately chilled on ice for 5 min. Samples were run on the gel in running buffer composed of 1x MOPS in dH_2O: the gel was run at 100V for 30 min followed for 4 hours at 120V. A peristaltic pump was used after 30 min of electrophoresis to allow for the re-circulation of buffer. The gel was washed for 20 min in 10x SSC, with gentle shaking, before RNA was transferred by Northern blot: First, 3MM paper, cut to a larger size than the gel, was placed on a glass sheet placed across a large tray containing 10x SSC, so that the paper acted

as wick for the absorption of SSC. The top of the paper was also thoroughly wetted and air bubbles released using a plastic pipette. The gel and then a piece of pre-soaked nitrocellulose membrane filter (GeneScreen Hybridisation transfer membrane, NEN), cut to the same size as the gel, were placed on top of the paper and the support, ensuring there were no air bubbles. Approximately 8 sheets of pre-soaked 3MM paper and of dry 3MM paper, cut exactly the same size as the nitrocellulose filter, were placed on top of the gel, followed by paper towels to a height of 10cm. Following this, the procedures were the same as those for Southern blotting (section 2.7.2)

2.8.4 Northern Blot Hybridisation

The procedure for hybridisation of DNA probe was as described for Southern hybridisation (see section 2.7.3). Following hybridisation and analysis, the blot was washed for 30 min at 80°C in stripping buffer (2mM EDTA, 1% SDS) in order to remove the bound probe, prior to rehybridisation with subsequent probes.

For the assessment of transgene expression in different tissues from TRAP-c-*fos*LTR mice, poly $(A)^+$ RNA extraction (using oligo d(T) column) and Northern blot analyses using 10μg mRNA per lane were carried out as described above. The v-*fos* was used as a probe for c-*fos* expression. This probe also hybridises to *fox* mRNA, a constitutively expressed mRNA in mouse tissues that can be used to normalise for loading. In addition, the blots were probed for c-*jun*, *fra*-1 and matrix metalloproteinase 9 (MMP-9) transcripts (section 2.3.5.1 for fragment preparation).

c-*fos* transgene expression was also assessed in KT1.5 clones. Cells were cultured in the presence and absence of Tc (0, 10μg/ml) for 3-4 days in 15mm dishes until almost confluent. Poly $(A)^+$ RNA extraction (using oligo d(T) column), Northern blot analyses and a v-*fos* DNA probe were used as previously described. RNA extracted from pJMF2-c-*fos* transfected ATDC5 cells (DT12.4) was kindly donated by Dr. D. Thomas and used as a positive control for transgene expression.

To evaluate the endogenous expression of *MSX2* in SaOS-2 cells, cells were plated in 15mm dishes and cultured until almost confluent before treatment with PTH (fragment 1-34; 0, 10, 100nM) for 2, 4 and 24 hours. Poly $(A)^+$ RNA extraction (using the Promega oligo d(T) column polyATtract isolation kit) and Northern blot analyses using

10μg mRNA per lane were carried out as described above using DNA probes containing *Msx2* coding sequence, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control for mRNA loading, and alkaline phosphatase was used as a control for PTH activity (section 2.3.5.1 for DNA fragment preparation).

2.8.5 RT-PCR

Total RNA was extracted from MC3T3-E1 and KT1.5 cells differentiated for assessment by RT-PCR. Wild type (wt) MC3T3-E1 cells and KT1.5 cells (c-Fos clone) were cultured overnight at $2x10^4$ cells/well of 24-well plates in α-MEM containing 10% FCS (Meldrum, Hants, UK), and in the presence of Tc (1μg/ml) for KT1.5 cells, to allow cells to settle and attach. Cells were washed with PBS and culture medium was changed to differentiation medium containing 10% batch-tested serum (Summit, Denver, CO, USA), ascorbic acid (0.1mg/ml) and β-glycerophosphate (10mM) in the presence and absence of Tc (KT1.5 cells) and BMP-2 (100ng/ml). Differentiation medium was changed every 3-4 days. Total RNA was extracted from cells in culture at day 1, 15, 30 (MC3T3-E1 cells) and 5, 16, 30 (KT1.5 cells) as described in section 2.8.2.

Primers for RT-PCR were obtained from Gibco BRL and the sequences of the primers were as follows:

<u>GAPDH</u>

Anti-sense	5'-GAT GCA GGG ATG ATG TTC-3'
Sense	5'-CCA CGA GAA ATA TGA CAA-3'
Product size	250kb

<u>Alkaline phosphatase</u>

Anti-sense	5'-TTC TGC TCA ATG GAC GCC GTG AAGC -3'
Sense	5'-GCA GGA TTG ACC ACG GAC ACT TATG -3'
Product size	409bp

RT-PCR (reverse transcription-polymerase chain reaction) was performed on the samples using a Gene AMP® Thermostable r*Tth* Reverse Transcription RNA PCR Kit (Perkin-Elmer) according to the manufacturers instructions. Briefly, the reverse transcription (RT) of 250ng total RNA into cDNA was performed by adding 5 units of r*Tth* DNA polymerase to the reverse transcription master mix which included 250ng total

RNA, 1x reverse transcriptase buffer, 1mM $MnCl_2$, 200μM dNTPs, 1.5μM anti-sense primers and dH_2O so that the total volume was 20μl. To control evaporation or refluxing, the mix was overlaid with 50μl of mineral oil (Sigma). Samples were incubated at 70°C for 15 min in a Stratagene® RoboCycler® Gradient PCR machine using thermosensitive PCR tubes.

The samples were maintained at 6°C while the PCR master mix was added which included 1x chelating buffer, 1.5mM $MgCl_2$, and 0.3μM sense primers, made up to a final volume of 100μl with dH_2O. PCR was performed as follows: After 3 min of preincubation at 95°C, amplification was performed for 30 cycles (MC3T3-E1 cells) or 24, 28, 30, and 32 cycles (KT1.5 cells) consisting of 1 min denaturing at 95°C, 1 min of annealing at 70°C and 1 minute of extension at 60°C. The final cycle had a higher extension time of 10 min at 60°C. Negative controls were included alongside all reactions to check for contamination, which contained all of the components of the reaction except RNA.

2.9 Protein extraction and Western hybridisation analysis

2.9.1 Protein extraction and SDS-PAGE

Total cellular protein was extracted from cells for use in Western blotting. Briefly, cells were washed in ice-cold PBS, 800μl Tween lysis buffer (TLB: 50mM HEPES, 2.5mM EGTA, 1mM EDTA, 150mM NaCl, 0.1% Tween-20, 1mM NaF, 1mM $NaVO_4$, 30mM β-glycerophosphate, 1mM DTT, 100μg/ml PMSF, 1μg/ml leupeptin, pepstatin and aprotinin, pH8) was added to the culture dish (15mm dish) and cells were scraped from the dish into a pre-chilled eppendorf tube. Cells were lysed by one freeze (dry ice)/thaw (on ice) cycle and with sonication (2x 15 sec). Following centrifugation (10,000 rpm at 4°C for 10 min), the protein concentration of the soluble fraction was determined with the BCA protein assay (see section 2.9.4) and the cell lysate was stored at –80°C.

Protein sample (30-50μg) was size fractionated using SDS-PAGE (Flowgen, Proseive) and electroblotted onto nitrocellulose membranes (GeneScreen Hybridisation transfer membrane, NEN). To make 20ml of a 8% resolving SDS-PAGE gel 2.85ml

dH_2O, 0.8ml Acrylamide/bis, 1.25ml Tris-HCl (1.5M, pH8.8), 50µl 10% SDS, 50µl 10% APS, and 2.25µl TEMED (Sigma) were mixed together. This was immediately poured between clean, pre-assembled SDS-PAGE plates and overlaid with H_2O-saturated butanol and allowed to polymerise for 20 min. The H_2O-saturated butanol was removed and the gel surface washed well with dH_2O. The stacking gel, which consisted of 3.1ml dH_2O, 500µl Acrylamide/bis, 1.25ml 0.5M Tris-HCl (pH6.8), 50µl 10% SDS, 100µl 10% APS, 5µl TEMED, was poured over the top and a clean comb inserted before the gel had polymerised.

Protein samples were mixed with 3x loading buffer (185mM Tris-HCl pH6.8, 0.45M mercaptoethanol, 6% SDS, 0.1% Bromophenol Blue, 30% Glycerol) at a ratio of 2:1. Samples, along with a protein molecular weight marker (NEB), were then heated to 100°C for 5 min, quenched on ice for 2 min, pulsed 10,000rpm, vortexed and then re-pulsed before loading on the gel. The gel was run at 8mA in electrophoresis buffer (25mM Tris-base, 250mM glycine pH8.3, 0.1% SDS) until the blue dye front entered the running gel, then at a constant 130V until the blue dye had migrated to the bottom of the gel.

2.9.2 Western blotting

Six pieces of 3MM Whatmann paper, cut to a size just larger than the resolving gel, were soaked in transfer buffer (129mM glycine, 25mM Tris-base, 10% methanol). One piece of nitrocellulose transfer membrane, cut to the size of the resolving gel, was first soaked in 100% methanol for 15 sec, then in dH_2O for 5 min, and finally equilibrated for at least 5 min in transfer buffer.

The membrane was placed on top of three pieces of pre-soaked filter paper. The gel was placed on top of the membrane and the three remaining pieces of pre-soaked filter paper were placed on top of this. Any air bubbles were smoothed out following each layering. This was placed onto a transfer cassette holder, which was closed and placed in a transfer tank so that the side of the cassette holder with the gel was facing the cathode. Transfer was allowed to proceed in transfer buffer for 1 hour at maximum voltage and 200mA.

To visualise the protein after transfer was completed, the membrane was soaked in methanol for 15 sec and then left to dry for 30 min on a piece of filter paper. The dry blot was immersed in 20% methanol in dH_2O for 2 min, then placed on a light box for transillumination of bands and photography. Following this, the blot was first placed in 100% methanol for 20 sec, then in dH_2O for 5 min, followed by TBS for 5 min.

2.9.3 Protein detection

Blots were blocked overnight at 4°C in 5% milk powder (Sainsbury's dried milk) in TBS. The blot was washed in TBST (TBS containing 0.1% Tween-20) for 5 min. Primary antibody incubations (c-Fos, Bcl-2, Bax, β-actin; see below) were performed in 1% milk powder in TBST for 1 hour at room temperature (or overnight at 4°C for c-Fos). The blot was rinsed briefly in TBST, then washed for 15 min and twice for 5 min in TBST.

The membrane was incubated with the appropriate horseradish peroxidase (HRP) conjugated rabbit secondary antibody (DAKO, Denmark) diluted at 1:2000 in 1% milk/TBST for 1 hour at room temperature on a rocking platform. The membrane was then briefly rinsed twice in TBST followed by one wash for 15 min and 2 washes for 5 min again in TBST. Protein bands were visualised with the ECL Western blotting detection system (Amersham Pharmacia Biotech) following manufacturer's instructions. Bands were visualised by placing a sheet of film (Kodak) on top of the membrane and exposing it at different time points (usually 10 sec, 40 sec, 2 min, 5 min).

For this thesis, the following primary antibodies (obtained from Santa Cruz Biotechnology, CA, USA) were used: c-Fos rabbit polyclonal IgG (diluted 1:1000), Bcl-2 rabbit polyclonal IgG (1:1000), Bax rabbit polyclonal IgG (1:5000), and β-Actin goat polyclonal IgG (1:2000).

For the assessment of c-Fos protein in KT1.5 clones, cells (KT1.5, 1.14, 1.20, 1.22) were cultured in 0, 1, or 10μg/ml Tc for 4 days before protein extracts were prepared and 30μg of protein was separated on SDS-PAGE (8%) gels as described above. A chondrogenic ATDC5 c-Fos over-expressing clone, DT8.6, was used as a positive control for c-Fos protein. Western blots were probed with a c-Fos specific antibody

(specific band ~ 62 kDa in size). In addition, blots were probed with β-actin antibody (~42 kDa) to control for loading.

To assess for Bcl-2 and Bax protein following induction of apoptosis in the presence of exogenous c-Fos in KT1.5, KT2.1, and AT9.2 cells, cells were cultured ± Tc for 48 hours to induce c-Fos expression, followed by either 10% serum or 0% serum (0.1% BSA) in α-MEM to induce apoptosis for 3 days. Protein extracts were prepared and 50μg of protein was separated on SDS-PAGE gels. An 8% gel was made to assess for c-Fos and β-Actin protein and a 12% gel for Bax (23 kDa), Bcl-2 (29 kDa) and β-Actin. Protein from the P1.7 osteoblast-like cell line, derived from the bone tumours of H2-c-*fos*LTR mice (Grigoriadis *et al*, 1993), was included as a positive control.

2.9.4 Estimation of protein concentration

For the determination of protein concentration in lysates prepared in lysis buffers for Western analysis, Luciferase assays and alkaline phosphatase assays (sections 2.9.1, 2.6.6.4, 2.6.9, respectively), the BCA (Bicinchoninic acid) protein assay reagent (Pierce, Rockford, Illanois, USA) was used according to manufacturer's instructions. For extracts prepared for the assessment of cAMP following PTH treatment (section 2.6.12) protein concentrations were also estimated using the Bio-Rad *DC* Protein Assay following the manufacturer's instructions.

2.10 *In Situ* Hybridisation

All stock solutions (e.g. dH_2O, PBS) were treated with 0.01% DEPC as described in section 2.8. Washes and incubations were performed at room temperature with gentle agitation, unless otherwise stated.

2.10.1 Riboprobe synthesis

Preparation of plasmid DNA for the production of the sense and anti-sense RNA probes was as described in section 2.3.5 and 2.3.5.1. Following purification of DNA, Digoxigenin (DIG) or [^{35}S]-dUTP incorporated riboprobes were prepared by *in vitro* transcription as described below.

2.10.1.1 Digoxigenin (DIG) RNA labelling

The DNA template (1-2µg) was incubated at 37°C for 2 hrs with 2µl of 10x Transcription buffer (Promega), 2µl of 10x DIG RNA Nucleotide mix (10mM GTP, ATP, and CTP, 6.5mM UTP, 3.5mM digoxigenin UTP; Roche), 2µl 0.1M DTT, 1µl (26 units) of RNase inhibitor (Promega), 2µl (40 units) of either T3, T7, or SP6 RNA polymerase (Promega) and dH_2O added to a final volume of 20µl. The DNA template was removed by the addition of 2µl RNase-free DNase 1 (10units/µl) and incubated for a further 15 min at 37°C. The RNA transcripts were precipitated by the addition of 2.5µl 4M lithium chloride and 75µl of ethanol at -20°C overnight. The RNA was pelleted by centrifugation at 4°C for 20 min, washed in 70% (v/v) ethanol in depc-dH_2O, air dried and resuspended in 20-100µl depc-dH_2O and 1µl RNase inhibitor (Promega). Riboprobes were briefly vortex and dissolved for 20 min at 37°C. A sample of the riboprobe (1-10µl) was then analysed on a gel as above to determine the quantity of riboprobe synthesised.

2.10.1.2 [^{35}S]-dUTP riboprobe synthesis

Riboprobes were produced according to the following Promega protocol. Linearised DNA template (2-3µg) was mixed with 5µl 5x Transcription Buffer (Promega), 0.5µl 1M DTT (BDH), 1.2µl 10mM GTP, 1.2µl 10mM ATP, 1.2µl 10mM CTP, and 50µM UTP and 7µl 10mCi/ml [^{35}S]-UTP (ICN Flow). Depc-dH_2O, RNA Polymerase (T3, T7 or SP6; Promega) and 1µl RNase inhibitor (Promega) were added to make a total volume of 25µl and incubated at 37°C for 1 hour and 40 min. To this reaction, 0.5µl DNase (Promega) was added together with 0.5µl tRNA (10mg/ml), 0.5µl 1M DTT and 0.5µl RNase inhibitor (Promega). This was incubated for a further 10 min at 37°C. The riboprobe was precipitated at -80°C for 1 hour or overnight by adding 300µl 100% ethanol, 10µl 3M NaOAc pH5.2, 1M DTT and 95µl depc-H_2O to the reaction mixture. Following centrifugation at 13,000rpm the riboprobe pellet was washed with 10mM DTT in 70% (v/v) ethanol twice, air dried for 10 min and re-suspended in 50µl 50mM DTT in depc-H_2O.

2.10.1.3 Hydrolysis of riboprobes

Labelled riboprobes (50µl) that were greater than 300bp in length were hydrolysed by addition of 50µl hydrolysis buffer (40mM $NaHCO_3$, 60mM Na_2CO_3 pH10.2, 0.1M DTT) at 60°C for *t* min where:

$$t = (L_o - L_f) / K \times L_o \times L_f$$

L_o = plasmid insert length (kb), L_f = length of riboprobe required (optimum riboprobe length for tissue penetration is 0.3kb), and K= hydrolysis constant for the hydrolysis buffer used = 0.11.

Hydrolysis was stopped by the addition of 6µl 3M ammonium acetate pH5.2, 3µl glacial acetic acid and 137µl ethanol. The hydrolysed probe was precipitated at -80°C for at least 1 hour or overnight before centrifugation at 13,000 rpm for 15 min to produce a pellet. The pellet was washed twice in 10mM DTT in 70% (v/v) ethanol, air dried for 10 min and resuspended in 50µl 50mM DTT in depc-H_2O.

2.10.2 DIG *in situ* hybridisation

Briefly, slides were pre-warmed to 50°C before they were dewaxed using Histoclear. Sections were rehydrated through a decreasing series of ethanol in depc-dH_2O (100, 95, 80, 60, 30% v/v) and finally depc-dH_2O, post-fixed for 20 min in 4% PFA in PBS and washed thrice in PBS to ensure removal of PFA. Sections were treated with 0.1M HCl, followed by three PBS and one depc-dH_2O wash. Sections were pre-equilibrated in digestion buffer (20mM Tris-HCl, 2mM $CaCl_2$ pH7.4) followed by digestion for 15 min (30 min for sections from decalcified tumours of TRAP-c-*fos*LTR mice) at 37°C with proteinase K (10µg/ml in digestion buffer). Proteinase K was washed away with three PBS washes before sections were fixed again in 4% PFA in PBS and washed three times in PBS. Sections were acetylated with freshly prepared 0.25% v/v acetic anhydride in 0.1M triethanolamine, pH8 for 20 min with continuous stirring and finally washed (thrice) with PBS. The area of slide not covered with section was carefully dried with clean tissues so that paraffin wax borders (using a DAKO paraffin pen) could be made around the sections. Pre-hybridisation buffer (100µl) containing 50% formamide (Sigma), 1mg/ml BSA, 0.02% (w/v) Ficoll, 0.02% polyvinylpyrrolidine, 5x SSC, 250µg/ml yeast tRNA, was carefully applied to each section. Slides were put on glass

rods in a hybridisation box lined with tissues saturated with 5x SSC and 50% v/v formamide and sections were pre-hybridised for 2-4 hours at room temperature.

DIG-labelled riboprobes were added to hybridisation solution (at approximately 20-200ng/ml). This was denatured at 85°C for 5 min and incubated on ice, before 50-70µl was added to each section and covered with parafilm cut approximately to the size of the sections. Following 5 min incubation at 80°C and cooling to hybridisation temperature, hybridisation was carried overnight at 50-70°C. After hybridisation, the tissue sections were washed with a series of washes; once for 5 min in 5x SSC at hybridisation temperature; 1 hour in 0.2x SSC at hybridisation temperature, followed by 10 min cooling to room temperature in 0.2x SSC.

The DIG hybridisation signal was detected using the Nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) substrate solution according to manufacturer's (Roche) instructions. Briefly, slides were washed in B1-DIG (100mM Tris, 150mM NaCl, pH7.5); followed by incubating for 1 hour in B2-DIG consisting of 1% Blocking reagent (Roche) in B1-DIG; then incubated for 1 hour with anti-DIG Fab-fragment alkaline phosphatase conjugate (0.2% v/v in B2-DIG). Unbound antibody was washed off by gentle shaking for 15 min (twice) in B1-DIG. Sections were equilibrated in B3-DIG (100mM Tris, 100mM NaCl, 50mM $MgCl_26H_2O$, pH9.0), before B3-colour (0.338mg/ml NBT, 168mg/ml X-phosphate/BCIP, 0.25mg/ml Levamisole, in B3-DIG) was added and allowed to develop (10 min to 72 hours) in the dark. The reaction was stopped by washing sections in B4-DIG (10mM Tris, 1mM EDTA, pH8.0). If the hybridisation signal was low, sections were lightly counter-stained with Haematoxylin and mounted in Aquamount with glass coverslips (Chance).

2.10.3 [^{35}S]-dUTP *in situ* hybridisation

The prehybridisation treatments were similar to DIG *in situ* hybridisation. Briefly, slides were dewaxed in Histoclear and rehydrated through the ethanol series as described above. Slides were rinsed twice in depc-dH_2O, followed by immersion in 0.2M HCl (20 min) and 2x SSC (5 min). Sections were treated for 15-30 min at 37°C with 10µg/ml proteinase K in 100mM Tris-HCl, 50mM EDTA, pH8.0; washed with 0.25% w/v glycine in PBS (2 min); and post-fixed in 4% PFA in PBS for 15 min. PFA was removed

by two washes in PBS and sections were acetylated for 10 min as described for DIG *in situ* hybridisation. Sections were washed in PBS before dehydration through ethanol series and left to air dry wrapped in tissue paper for at least 1 hour.

Probes were diluted in hydridisation buffer: 50% formamide, 10% (w/v) dextran sulphate, 50mM DTT, 1mg/ml BSA, 0.02% (w/v) Ficoll, 0.02% polyvinylpyrrolidine, 50µg/ml poly A RNA, 500µg/ml yeast total RNA, 0.3M NaCl, 10mM Tris (pH7.4), and 0.5 M EDTA. This was denatured at 70°C and cooled on ice, before 70µl was added to each pre-treated section and covered with parafilm. Hybridisation was carried out at 50-65°C overnight in a sealed hybridisation box containing 50% formamide, 5x SSC soaked tissues.

After hybridisation slides were quickly transferred to a rack for the following 15 min washes (2x SSC, 50% formamide, and 10mM DTT): one at 55°C with agitation, during which time the coverslips were flushed off; another at 55°C; and then one at 65°C. Slides were equilibrated at 37°C for 15 min (twice) in RNase buffer (500mM NaCl, 10mM Tris-HCl, pH8.0, 1mM EDTA), before treatment with 20µg/ml RNase A in RNase buffer for 30 min at 37°C. Slides were washed in RNase buffer to remove RNase A (15 min, 37°C), before being washed twice in 2x SSC, 50% formamide, 10mM DTT at 65°C for 20 min; once in 0.1x SSC, 10mM DTT at 65°C for 20 min; and 5 min at room temperature in 0.1x SSC. Slides were dehydrated in 70% ethanol (v/v) with 0.3M ammonium acetate, 95% ethanol (v/v), and finally 100% (v/v) ethanol for 2 min each and air-dried.

Slides were exposed to X-ray film (Kodak) overnight to examine both the quality of hybridisation and to estimate time of exposure for autoradiography. Subsequently slides were dipped in Ilford nuclear K5 emulsion gel, melted at 40°C and diluted 1:1 with dH_2O containing 2% v/v glycerol. Slides were left to air dry for 2 hours in a light-tight box. The slides were exposed at 4°C (in the dark in a light-tight and air-tight box containing silica gel crystals) for approximately 10-14 days depending on radioactive signal. Before being developed, slides were prewarmed to room temperature, then immersed in Kodak D19 developer for 5 min at 18°C, rinsed in dH_2O (2 min), and fixed for 5 min in ammonium thiosulphate. Slides were washed for 15 min in the dark, under

running tap water followed by two washes in dH_2O. Sections were counterstained with Haematoxylin and Eosin, dehydrated and cleared in Histoclear before being mounted using DPX mounting medium with glass coverslips.

2.11 Statistical analysis

All experiments were repeated in triplicate unless otherwise stated. All data are presented as means ± S.D. In all cases, differences between groups were analysed by Students t-test: *(or $^{\#}$) $P<0.05$; **(or $^{\#\#}$) $p<0.01$; ***(or $^{\#\#\#}$) $p<0.001$.

2.12 Photography

Slides were analysed using an Olympus BH-2 photomicroscope with phase contrast and dark field optics. Photography was done under both dark and light field, using an Olympus system 2 camera and Fujichrome or Kodak 64T film at an ISO setting of 64. Fluorescence microscopy was carried out using photomicroscope, at an ISO setting of 3200, using Fuji 1200 asa film speed. Slides were converted to colour prints using Printscan on Adobe photoshop.

Northern and Southern blots were scanned directly into Adobe photoshop using an Epson Flat bed scanner.

3. A putative role for c-Fos in the pathophysiology of Paget's Disease

3.1 Introduction

Paget's disease of bone is a chronic skeletal disorder characterised by areas of rapid bone turnover, deformity and degeneration. Patients with the disease have an increased risk of malignant transformation, and are more likely to develop osteosarcomas (Freemont, 1996). The primary lesion in Paget's disease is thought to involve bone-resorbing osteoclasts, which are increased up to 50 to 100-fold both in number and multinuclearity, resulting in an increase in bone turnover. The specific causes of the apparent increase in osteoclast activity, in addition to the molecular mechanisms underlying cellular transformation and oncogenesis are poorly understood.

Several candidate factors have been associated with Paget's disease (reviewed by Gehron-Robey and Bianco, 1999): For example, the cytokine IL-6 is apparently up-regulated specifically in Pagetic osteoclasts, which constitutively express IL-6 receptors and the transcription factor NF-IL-6, suggesting a possible autocrine role for IL-6 (Hoyland *et al*, 1994; Reddy *et al*, 1999; for review, see also Mee and Sharpe, 1993). Paget's disease has a strong familial tendency, suggesting there is a genetic component to the disease (reviewed by Hul, 1999). Indeed, it has an inexplicable geographical distribution, with the disorder being found to be most common in Europe, North America, Australia, and New Zealand (Barker *et al*, 1984). The overall prevalence rates from radiology studies in hospitalised patients over 55 years found the highest frequency (4.6%) in England, particularly in the region of the industrial north (6.3% to 8.3%), and France (2.4%), and lower rates were observed in other Western European countries (e.g., 0.7-1.7% in Ireland, 1.3% in Spain and West Germany, and 0.5% in Italy and Greece). Similarly high rates were found in the older population in Australia and in New Zealand (3-4%) and North America (2-3%). Moreover, Paget's disease is a disorder of older people (aged 55 years or older), but which affects women and men equally.

A genetic aetiology of Paget's disease is supported by a study showing that 15% to 30% of patients have a positive family history of the disorder (Siris *et al*, 1991). Both Paget's disease and the Paget's-related disease Familial Expansile Osteolysis (FEO) have been mapped to a susceptibility locus on chromosome 18q in some families (Cody *et al*, 1997; Haslam *et al*, 1997; Hocking *et al*, 2000). Interestingly, this locus (termed PDB2 in Paget's disease) is in a candidate interval containing the

gene for RANK, the receptor found on osteoclasts and important for osteoclast differentiation. Recent work, however by Wuyts *et al* (2001) have discounted mutations in RANK or OPG as the possible cause of Paget's disease and recently, mutations affecting RANK have been demonstrated in patients with FEO (Hughes *et al*, 2000). Furthermore, there were several polymorphisms in the second intron of OPG, which need to be further investigated. An additional locus (PBD1) has been mapped on chromosome 6, with a possible association between Paget's disease and the HLA complex, and at least one other exists elsewhere in the genome (Fortino *et al*, 1977; Haslam *et al*, 1998). The anti-apoptotic gene Bcl-2 has also been mapped to 18q and recent preliminary data indicate that Bcl-2 expression is up-regulated in Paget's disease (Haslam *et al*, 1997; Cody *et al*, 1997; Mee, 1999). Pagetic osteoclasts also respond differently to osteotropic factors such as 1,25-$(OH)_2D_3$ and calcitonin. The increased sensitivity to 1,25-$(OH)_2D_3$ may be a result of increased expression of vitamin D receptor (VDR) mRNA or increased affinity for the VDR (Mee *et al*, 1996; Menaa *et al*, 2000). In addition, the presence of IL-6 may enhance the responsiveness of Pagetic osteoclasts to RANKL (Menaa *et al*, 2000), which is also unregulated in Pagetic osteoblastic stromal cells (Menaa *et al*, 2000; Neale *et al*, 2000). A viral aetiology has also been postulated, with a number of studies implicating paramyxoviruses including: measles, respiratory syncytial virus, simian virus 5, parainfluenza virus Type 3, and canine distemper virus (reviewed by Singer, 1999).

Previous expression studies in Pagetic osteoclasts has demonstrated that there is an increase in c-Fos expression (Hoyland and Sharpe, 1994); an observation that provides a possible explanation for the molecular basis of Paget's disease, including the potential for cellular transformation and oncogenesis. As stated in Chapter 1 (section 1.6.3 and 1.6.4), gain-of-function and loss-of-function studies have implicated c-Fos in the regulation of bone development and bone disease. c-Fos is associated with a variety of biological processes, ranging from transformation, to cell cycle progression and differentiation (Angel and Karin, 1991). Transgenic mice overexpressing c-*fos* using constitutive promoters (e.g., H2-c-*fos*LTR mice) develop remodelling osteosarcomas due to specific c-Fos-dependent oncogenic transformation of osteoblasts (Grigoriadis *et al*, 1993). Moreover, histopathological analysis revealed many features which resemble Paget's disease (Grigoriadis *et al*, 1993;

Ruther *et al*, 1989; Wang *et al*, 1992). In contrast, c-Fos knockout mice lack osteoclasts and develop the bone remodelling disease osteopetrosis (Wang *et al*, 1992; Grigoriadis *et al*, 1994), clearly demonstrating that c-Fos is an essential gene for osteoclast differentiation and bone remodelling in general (see also Grigoriadis *et al*, 1995 for review). Thus, the gain- and loss-of-function analyses have clearly implicated the c-Fos proto-oncogene and AP-1 transcription factor in osteoclast differentiation, bone cell transformation and tumour formation, all of which are features of Paget's disease.

To further examine the role of c-Fos in Paget's disease, and consistent with the observations that osteoclasts in Pagetic bone express high levels of c-Fos (Hoyland and Sharpe, 1994), the aim of this chapter was to generate transgenic mice in which c-*fos* is overexpressed specifically in osteoclasts using the promoter for tartrate-resistant acid phosphatase (TRAP). Some of the results from this work were published recently (Beedles *et al*, 1999).

3.2 Generation of TRAP-c-*fos*LTR transgenic mice

The murine TRAP-c-*fos*LTR construct was prepared by Dr. A.E. Grigoriadis and consists of a murine TRAP promoter (pBS-TRAP; obtained from Dr. G.D. Roodman, San Antonio; see Boyce *et al*, 1995) fused to a full length genomic sequence for the murine c-*fos* gene (Figure 3.1). This promoter was chosen because it is expressed at high levels in osteoclast precursors and differentiated osteoclasts, and it has been shown previously to function reliably for targeting transgenes to osteoclasts in mice (Boyce *et al*, 1995; Schwartzberg *et al*, 1997; Hentunen *et al*, 1997). The 3' mRNA destabilising sequences and polyadenylation (polyA) site of c-*fos* were replaced by a 3' long terminal repeat (LTR) from the FBJ-murine sarcoma virus. This modification is essential for ensuring stability of the exogenous c-*fos* mRNA (Ruther *et al*, 1987; Grigoriadis *et al*, 1993). This vector gives rise to two exogenous c-*fos* transcripts, a 3.0kb transcript, which terminates at the polyA site in the LTR and which encodes for the functional c-Fos protein, and a shorter 2.0kb transcript, which terminates at a cryptic polyA site present in the FBJ-derived sequence (Figure 3.1). The construct was microinjected (without plasmid sequences) into fertilised eggs by Dr. N. Faruque, followed by transfer into foster females.

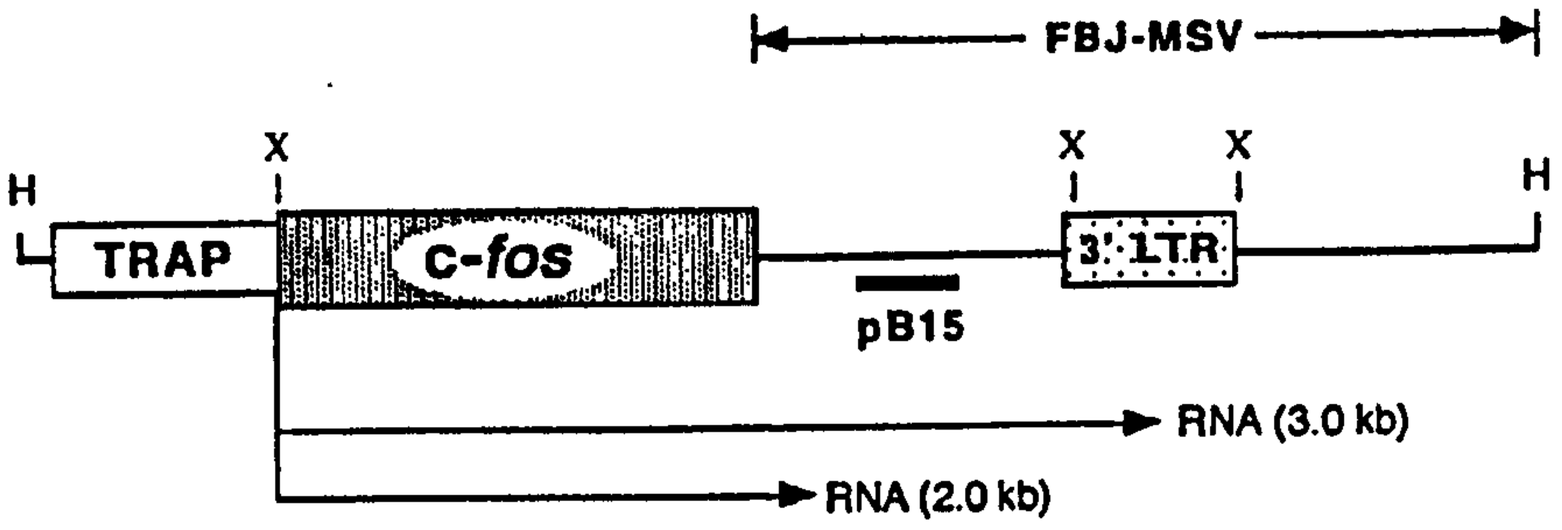


Figure 3.1 - The DNA construct (TRAP-c-*fos*LTR) used for the generation of TRAP-c-*fos*LTR transgenic mice. The murine TRAP promoter (pBS-TRAP; Boyce *et al*, 1995) was fused to a full length genomic sequence for the murine c-*fos* gene in which the 3' mRNA destablising sequences have been replaced by a 3' long terminal repeat (LTR) from the FBJ-murine sarcoma virus (Ruther *et al*, 1987). Two exogenous c-*fos* transcripts are synthesised as indicated. H – *Hind*III; X – *Xba*I. The location of the transgene specific probe (pB15) used for *in situ* hybridisation analysis has been depicted.

A total of nine founder animals were obtained which contained between ~5 and 50 copies of the transgene as determined by Southern blot analysis of tail DNA (see section 2.7). Of these, five developed noticeable swellings in the long bones between 3-6 weeks of age, and some mice were severely growth retarded. Radiographic analysis of phenotypic founder animals indicated the presence of marked skeletal abnormalities in virtually all bones, in contrast to the normal skeletal development in transgene-negative littermates (Figure 3.2). Two related phenotypes were observed in these five transgenic animals. Three founders displayed a severe sclerotic phenotype over the entire skeleton with long bones, vertebrae, pelvis and skull being severely affected (Figure 3.2 A). In the remaining two founder animals, skeletal lesions were detected which developed very rapidly into very large calcified tumours (Figure 3.2 B). Again, these lesions were present in most bones of the skeleton. All phenotypic mice developed these skeletal abnormalities shortly after birth (3-6 weeks post-natally), prior to reaching sexual maturity, and together with the size and severity of the lesions, attempts to breed them and obtain transgenic offspring were not successful. Nevertheless, the generation of five independent founders which developed similar phenotypes (see also below) clearly indicated that

the defects were independent of the transgene integration site, and suggested that exogenous c-Fos expression was causal in generating the skeletal abnormalities.

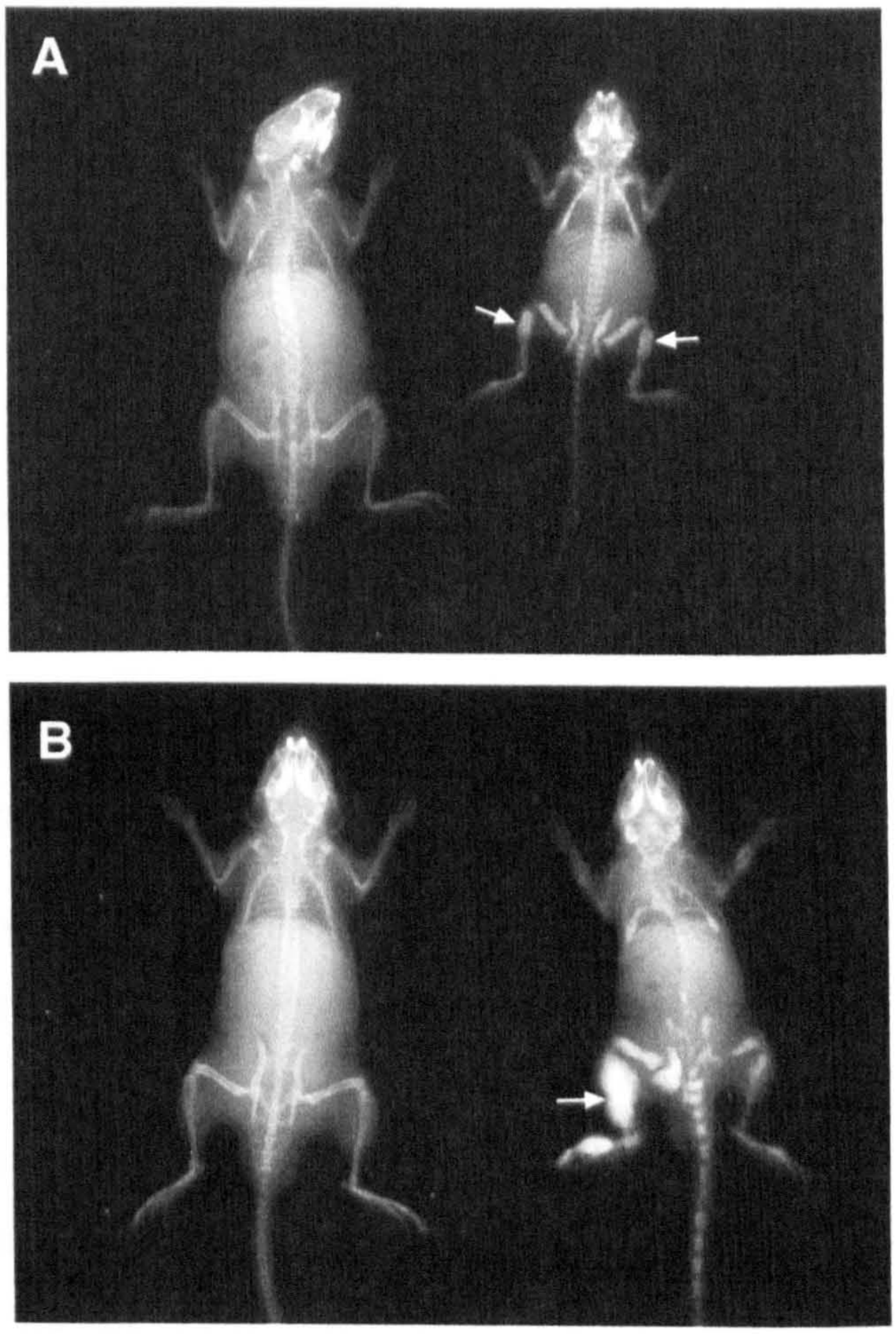


Figure 3.2. - Skeletal abnormalities in TRAP-c-*fos*LTR transgenic mice. Radiographic analysis of wild-type (left) and transgene positive (right) littermates, showing the severity of lesions in transgenic bone. (A) X-rays of a 5 week-old normal mouse (no. 228-2) and its age-matched transgenic littermate (no. 228-1) showing specific radio-dense lesions in all bones (arrows), including long bones, vertebrae, pelvis and skull. (B) X-rays of a 5 week-old normal mouse (no. 250-8) and its age-matched transgenic littermate (no. 250-9) showing large calcified tumours (arrow). Radiographs were prepared by Dr A.E. Grigoriadis.

From this work only single founder animals could be produced, such that different animals were used for different experimental procedures, as summarised in Table 3.1, and in the respective figure legends.

Table 3.1 – Phenotype of individual TRAP-c-*fos*LTR mice

Mouse number	Phenotype	Figure
228-1	Sclerotic	3.2 A
250-9	Calcified tumour	3.2 B
1766	Sclerotic	3.3 A
250-9	Calcified tumour	3.3 B
228-1	Sclerotic	3.4 and 3.5

3.3 Northern blot analysis of tissues from TRAP-c-*fos*LTR transgenic mice

To investigate whether there was a relationship between the development of the skeletal phenotypes in the founder transgenic mice and expression of the c-*fos* transgene, Northern blot analysis was performed on poly(A)$^+$ RNA isolated from tissue samples of normal and tumour tissues (see section 2.8). In one particular transgenic founder, exogenous c-*fos* was expressed in tumour-bearing bones and at lower levels in the heart, but not in any other tissues (Figure 3.3 A). Endogenous c-*fos* RNA was also expressed in the intestine and muscle, and at lower levels in brain and liver tissues. In a second independent founder, transgene expression was detected at high levels in lesional bone tissue, but also at lower levels in liver which did not develop any macroscopic abnormalities (data not shown). Significantly, Northern blot analysis of tissues from the four founder animals which did not develop any bone abnormalities failed to demonstrate expression of the c-*fos* transgene in tissues analysed (data not shown). These data therefore suggest that the expression of exogenous c-*fos* was likely to be responsible for the development of the observed skeletal lesions.

Further Northern blot analysis was carried out for another transgenic founder. As observed above, exogenous c-*fos* expression was confirmed in tumour-bearing bones, calvaria and the tumour itself (Figure 3.3 B), with no other tissues demon-

strating expression of the transgene. This Northern blot was also probed for other AP-1 family members. Northern blot analysis for c-*jun*, showed no change in expression (Figure 3.3 B). c-*jun* was ubiquitously expressed, with low levels discernible in long bone and tumour. In contrast, the Fos-related gene (*fra*-1) was expressed in long bone and tumour tissues of TRAP-c-*fos*-LTR mice (Figure 3.3 B) but is not reported to be expressed at high levels in adult wild type bone. These results are consistent with the fact that *fra*-1 is a Fos-responsive gene (Braselmann *et al*, 1992; Schreiber *et al*, 1997; Matsuo *et al*, 2000; Fleischmann *et al*, 2000; Jochum *et al*, 2001; see discussion below). Finally, expression analysis for MMP-9 (Gelatinase B), a marker for osteoclasts, showed that levels were specifically high in bone tissue and tumours from TRAP-c-*fos*LTR mice (Figure 3.3 B). This would suggest that there are many osteoclasts present in the tissue. This was later confirmed by TRAP histochemistry and *in situ* expression analysis for MMP-9 (see below).

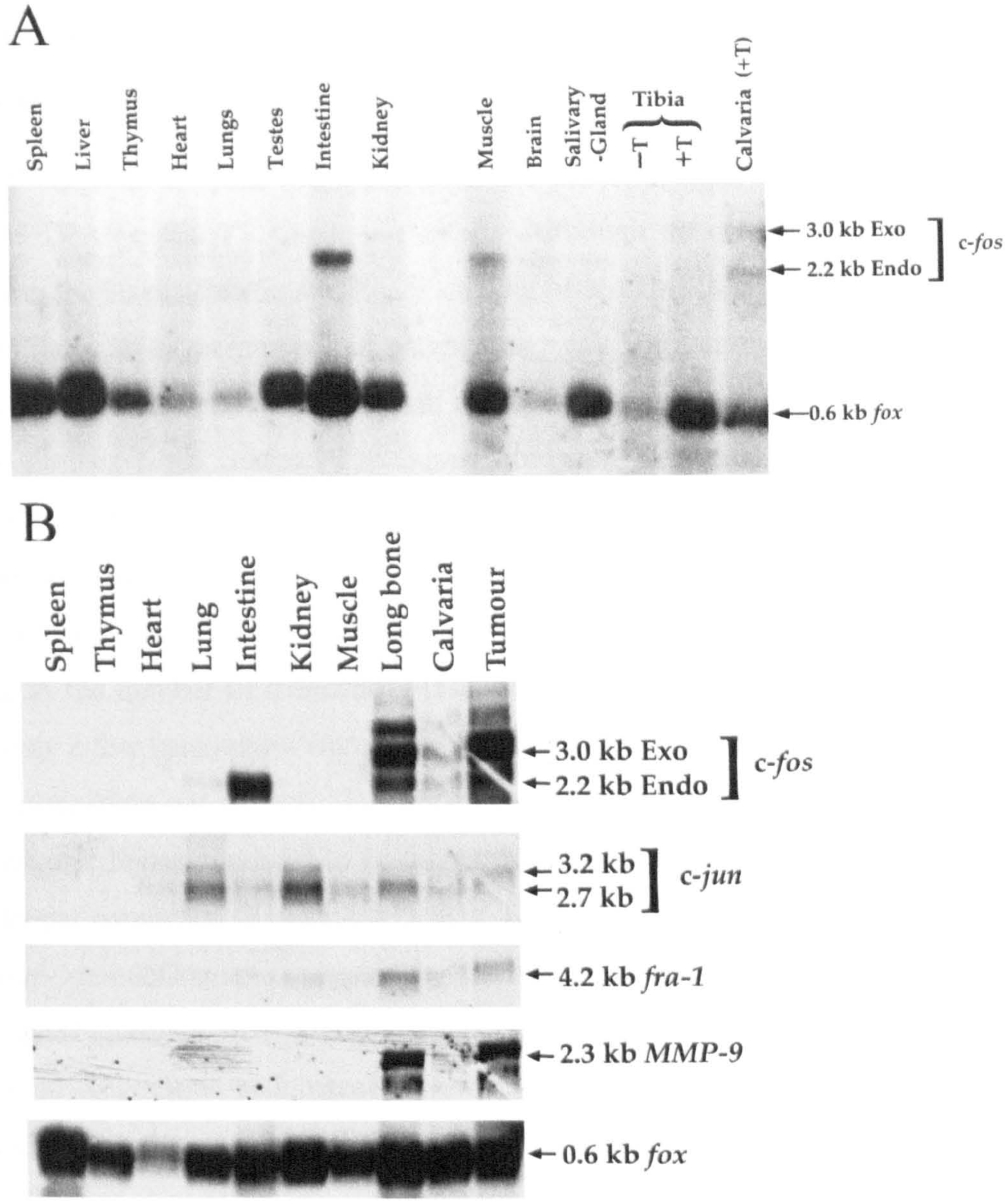


Figure 3.3 - Northern blot analysis of c-*fos* transgene expression in different tissues of a TRAP-c-*fos*LTR transgenic mice. (A) The Northern blot membrane from mRNA of one transgenic founder (no. 1766) was hybridised with a ^{32}P-labelled v-*fos* probe which detects both exogenous (3.0kb) and endogenous (2.2kb) c-*fos* transcripts (as described in section 2.8.4). The endogenous *fox* gene (0.6kb) was used as a control for RNA loading. The exogenous c-*fos* transcript was evident in tumour-bearing bones (+T) like tibia and calvaria, compared with no signal in bones lacking tumours (-T). (B) A second founder animal (250-9) was hybridised with the v-*fos* probe to confirm transgene was expressed especially in bones containing tumour. In addition, a probe for c-*jun* was used (3.2 and 2.7kb transcripts), which appeared to be ubiquitously expressed, but was low in bone and tumour. Furthermore transcripts for *fra*-1 (4.2kb) and *MMP*-9 (2.3kb) were found to be highly expressed (B).

3.4 Histopathological analysis of skeletal lesions from TRAP-c-*fos*LTR transgenic mice

Histopathological analysis was performed on sections from representative long bones of TRAP-c-*fos*LTR transgenic mice. Although no quantitative assessment was made, the Haematoxylin and Eosin staining of sections (section 2.5) revealed an apparent increase in the number of osteoclasts, with high numbers of nuclei. Also there was increased bone trabeculae and numbers of osteoblasts and a marked bone marrow fibrosis when compared to bones from non-transgenic littermates (Figure 3.4 A, B). Long bones in general also exhibited a poorly developed cortical bone (data not shown).

Histochemical staining for the osteoclast marker TRAP demonstrated a marked increase in the number of osteoclasts (Figure 3.4 C) compared to wild-type bones where only a few osteoclasts were observed primarily lining the distal endochondral growth plate (data not shown). Further examination, showed that the osteoclasts in the transgenic bones appeared to be larger than normal, containing a larger number of nuclei per osteoclast (Figure 3.4 D, E). The lesions appeared to undergo extensive bone remodelling as evidenced by the numerous reversal lines present in all trabeculae (Figure 3.4 D, E), which would indicate increased osteoclast activity. In addition to osteoclasts and osteoblasts, occasional pockets of chondrocytes were observed suggesting that chondrogenic cells or specific precursors were also affected (data not shown).

To confirm further the increase in number and activity of osteoclasts within these remodelling lesions, non-radioactive *in situ* hybridisation analysis was performed on adjacent sections of transgenic bones for matrix metalloproteinase-9 (MMP-9), as detailed in section 2.10, which is another specific marker for osteoclasts *in situ* (Reponen *et al*, 1994; Grigoriadis *et al*, 1994). The results suggested that all TRAP-positive cells were also expressing high levels of MMP-9 (Figure 3.4 F), which were large and contained numerous nuclei (Figure 3.4 G, H), confirming that these were *bona fide* osteoclasts.

To investigate the cell-specificity of c-*fos* transgene expression, non-radioactive and radio-active *in situ* hybridisation analysis was carried out with a transgene specific probe, pB15 (Figure 3.1). *In situ* hybridisation analysis indicated that the

transgene was expressed in the osteoclasts within the lesions (Figure 3.5 A-D). However, clearly not all osteoclasts appeared to express the transgene (Figure 3.5, A and C). In addition there was expression in non-osteoclastic cells presumably osteoblasts or osteoblast precursors (Figure 3.5 A and C). Furthermore, transgene expression was clearly evident in the osteoblasts of bone which had not yet progressed to tumour (Figure 3.5 D).

3.5 Immunohistochemistry of lesions from TRAP-c-*fos*LTR transgenic mice

To investigate the cell-specific expression of the c-Fos protein within these lesions, immunocytochemical analysis was performed on adjacent sections using a murine c-Fos polyclonal antibody (see section 2.5.4). The results indicated that high levels of c-Fos protein were detected in almost all osteoclasts lining the bone surfaces (Figure 3.5 E, F). In addition, c-Fos protein was expressed in some, but not all osteoblasts and chondrocytes (Figure 3.5 E, F) and in many fibroblastic connective tissue cells within these lesions (data not shown). It should be emphasised that the antibody used in these experiments detects both endogenous as well as exogenous c-Fos proteins, therefore it is perhaps not surprising that many cell types might be expressing c-Fos. Indeed, this is suggested by the Northern blot analyses where endogenous c-*fos* RNA was also detected within the bone lesions (Figure 3.3). Taken together, the features of the phenotype which develops in c-*fos*-overexpressing transgenic mice are an increase in the number and multinuclearity of osteoclast-like cells and the generation of lesions characterised by high rates of abnormal bone turnover.

Hoyland and Sharpe (1994) previously observed that high levels of c-Fos are also present in bone cells of patients with Paget's disease. To assess cell specificity, the expression of c-*fos* was investigated by radioactive *in situ* hybridisation techniques. They found high levels of c-*fos* expression in multinucleated osteoclasts in Pagetic bone, whereas c-*fos* expression in osteoclasts present in control osteophyte tissue was not detectable (see Hoyland and Sharpe, 1994; Beedles *et al*, 1999). In addition, the expression of c-*fos* mRNA was confirmed by immunohistochemical staining for c-Fos protein on adjacent sections (Hoyland and Sharpe, 1994). These data further implicate a role for c-Fos in the altered osteoclastic activity which is a

characteristic of Paget's disease. More importantly, the striking similarities between the morphology and activity of the osteoclasts present in Paget's disease compared with those which develop in TRAP-c-*fos*LTR transgenic mice, suggest that these mice might be useful for further delineating the molecular basis for the altered osteoclastic behaviour, and perhaps, the subsequent events that lead to osteosarcoma formation.

Previous work has suggested a role for Bcl-2 in normal and Pagetic osteoclasts (Selander *et al*, 1997; Lagasse and Weissman, 1997; Cody *et al*, 1997; Mee, 1999), with Bcl-2 message levels being upregulated in Pagetic osteoclasts compared to normal osteoclasts. Since apoptosis is the ultimate fate of the osteoclast, it is possible that the observed increase in size of the osteoclasts in TRAP-c-*fos*LTR mice may be a result of increased Bcl-2 expression and hence survival. To investigate the the expression of genes involved in apoptosis of the osteoclasts in TRAP-c-*fos*LTR mice, the expression of the anti-apoptotic protein Bcl-2 was studied by immunohistochemistry (section 2.5.4). Interestingly, there were high levels of Bcl-2 protein in the osteoclasts of these mice (Figure 3.5), compared to wild type osteoclasts (data not shown). Bcl-2 protein was clearly visualised around the ruffled border of these osteoclasts. Clearly, high levels of Bcl-2 protein were not observed in the osteoblasts of these lesions. Thus, Bcl-2 may be affording an anti-apoptotic function specifically in these osteoclasts, resulting in a prolongation of their existence and hence functionality (Lagasse and Weissman, 1997; Okahashi *et al*, 1998). It is not unlikely that the increased longevity of these osteoclasts is contributing to fusion potential and subsequent multinucleated phenotype and functionality observed in TRAP-c-*fos*LTR mice.

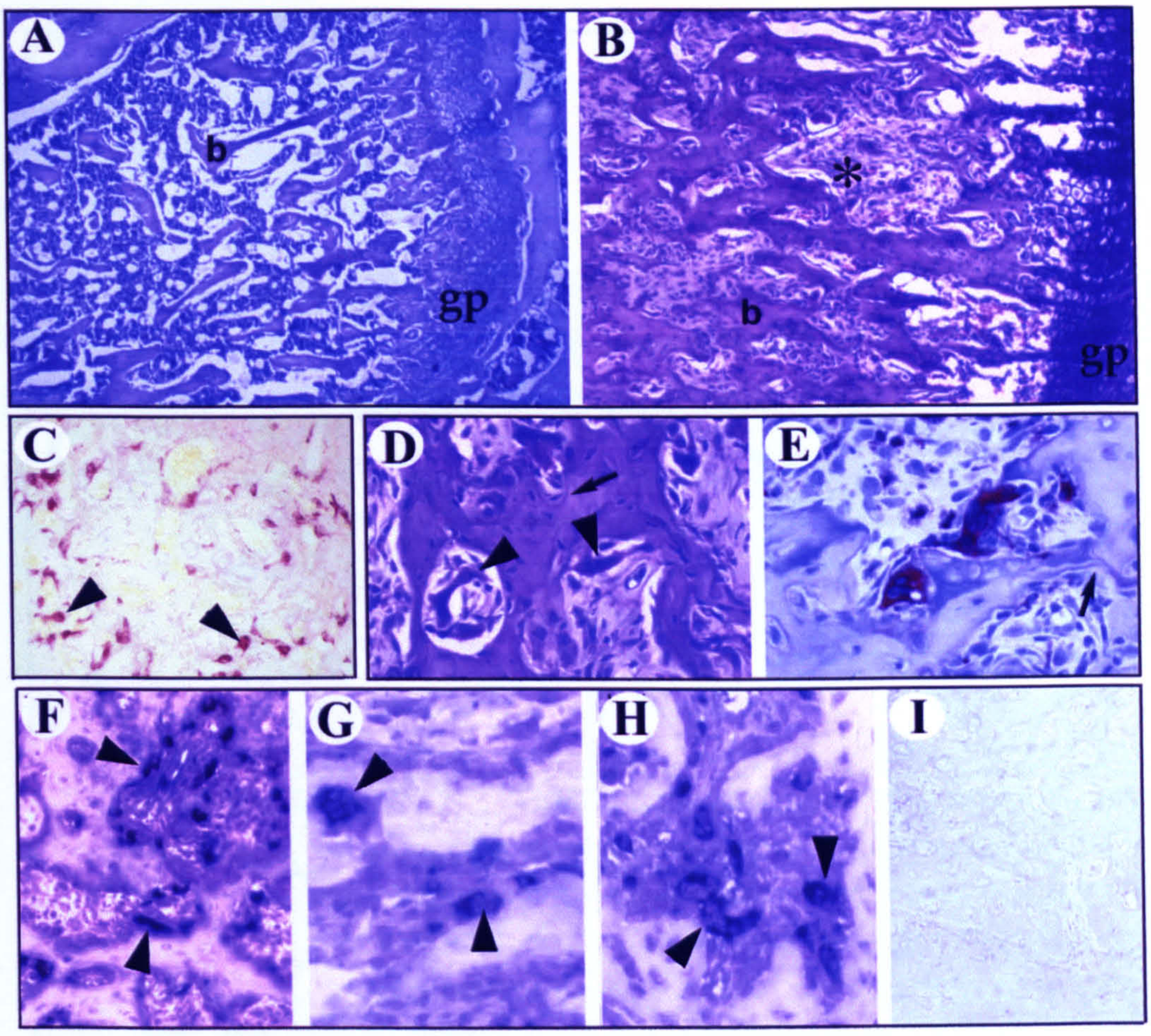


Figure 3.4 - Histological analysis of TRAP-c-*fos*LTR transgenic mice. Haematoxylin and Eosin stained sections of a typical wild-type tibia (A) and a TRAP-c-*fos*LTR transgenic tibia (B), demonstrating the presence of increased bone trabeculae in transgenic mice compared to non-transgenic littermates. The bone marrow space is largely fibrotic with little haematopoietic cell activity (*). TRAP histochemistry detected increased numbers of osteoclasts (C-E) compared to wild-type bone (data not shown). (C) Detection of TRAP-positive osteoclasts in adjacent sections to B, and (E) a high power view of multinucleated, TRAP-positive osteoclasts (counterstained with Haematoxylin). Arrows in D and E show reversal lines. Digoxigenin-labelled anti-sense riboprobes for MMP-9 detected high levels of expression of MMP-9 in TRAP-positive, multinucleated osteoclasts of TRAP-c-*fos*LTR transgenic mice (F, G, H). Control sense riboprobe hybridisations revealed no specific signal (I). All sections are 5μm paraffin sections prepared and stained as described in section 2.5. Arrowheads in all panels indicate osteoclasts. gp - growth plate, b – trabecular bone. A, B, I x100; C, F x200; D, E, G, H x400.

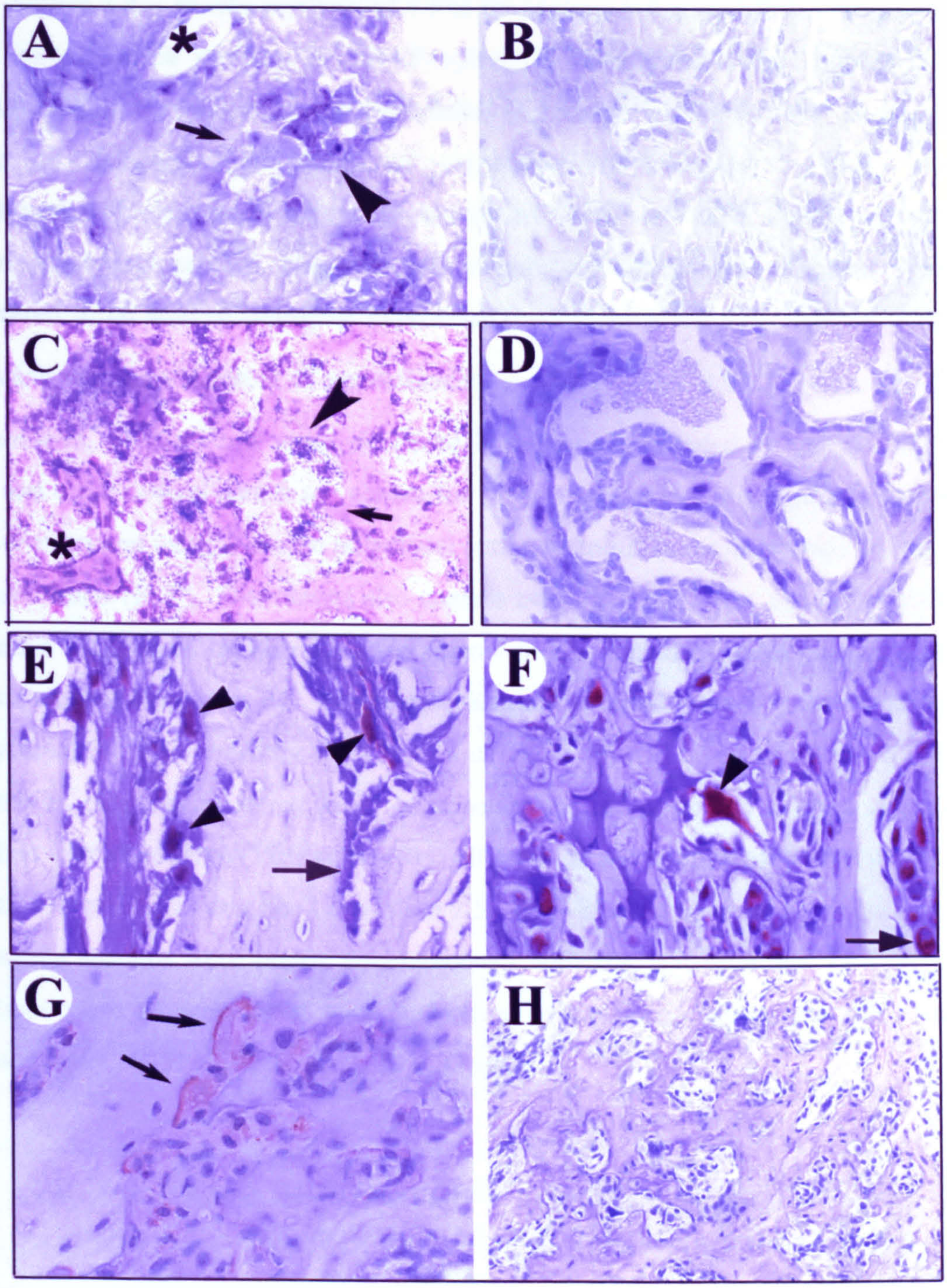

A
B
C
D
E
F
G
H

Figure 3.5 – *In situ* analysis of c-Fos transgene, c-Fos and Bcl-2 protein in the lesions which develop in TRAP-c-*fos*LTR transgenic mice. Digoxigenin (DIG)-labelled anti-sense (A) and ^{32}P-labelled (C) riboprobes for the B15 transgene sequence detected high levels of expression of transgene transcripts in multinucleated osteoclasts within the lesions of TRAP-c-*fos*LTR transgenic mice (arrowheads in A and C). However, occasionally osteoclasts were observed which appeared to be void of transgene expression (arrows in A and C). Transgene expression was also apparent in non-osteoclastic cells (* in A and C) which may be osteoblasts. DIG control sense riboprobe hybridisation revealed no specific signal (B) and sense control data is not shown for ^{32}P-labelled probes. Immunolocalisation of c-Fos protein using a murine polyclonal antibody confirms the expression of c-Fos in osteoclasts (E, F). Osteoblasts also express c-Fos in some areas, although clearly negative osteoblasts are also observed (arrow in E). Similarly, some chondrocytes express c-Fos (arrow in F). Immunolocalisation of Bcl-2 protein using a murine polyclonal antibody confirms high levels of Bcl-2 protein localised to the ruffled border of osteoclasts (arrow in E) of bone lesions, compared to control sections probed with goat serum/TBS alone (F). All sections are 5μm paraffin sections prepared and stained as described in section 2.5. DIG-labelled sections and immunolocalisation sections were briefly counterstained with Haematoxylin and ^{32}P-labelled sections were counterstained with Haematoxylin and Eosin before mounting and photography. C, H x200; A, B, D, E, F, G x400.

3.6 Discussion

In this chapter the consequences of ectopic c-Fos expression in mature osteoclasts have been investigated in an attempt to mimic the high levels of c-Fos which are observed in Pagetic osteoclasts (Hoyland and Sharpe, 1994). While earlier c-Fos knockout experiments have demonstrated unequivocally that c-Fos must be expressed in osteoclast progenitors for normal differentiation (Grigoriadis *et al*, 1994), it was not possible in that study to discern whether c-Fos is also required for the fusion and activity of fully differentiated, multinucleated osteoclasts. This study has shown that targeting c-*fos* expression to osteoclasts under the control of the osteoclast-specific TRAP promoter (TRAP-c-*fos*LTR mice) results in the rapid development of highly remodelling lesions and formation of bone tumours. Five independent transgenic founder mice were identified which expressed exogenous c-Fos but which unfortunately could not be bred to generate individual families of transgenic mice due to the severity and early onset of the phenotype. Thus, while the absolute causal role of c-Fos in the development of these phenotypes is difficult to prove by analysing only founder animals, it is clear that the observed skeletal defects were very specific to c-Fos overexpression and independent of the transgene integration site.

The phenotype amongst affected animals, such as the time of onset, the histological characteristics, the cellular composition, etc., were remarkably similar in all bones analysed from all founder mice, suggesting that the defects were likely to be caused by ectopic c-Fos expression. Notably, all lesions contained numerous osteoclasts, most of which contained a very large number of nuclei when compared to osteoclasts in wild-type littermates, and in this regard these lesions exhibit some similarities to Paget's disease. The osteoclasts expressed high levels of TRAP activity in addition to MMP-9, which is a reliable marker for osteoclasts *in situ*. Furthermore, preliminary analysis using *in situ* hybridisation has also found similar levels of cathepsin K in the osteoclasts of these lesions (data not shown), further supporting the osteoclasts' functionality.

From these findings, it is tempting to speculate that the consequences of this increased osteoclast number and activity in TRAP-c-*fos*LTR mice are the consequential stimulation of a cascade in which there is an increase in the recruitment and dif-

ferentiation of osteoblasts, which leads to a highly remodelling lesion in which osteoclastic bone resorption and osteoblastic bone formation are accelerated, causing bone deformation. Despite the fact that the initial appearance of some of the skeletal defects (e.g., as in Figures 3.2 A, 3.4 B) might suggest an osteopetrotic phenotype, implying that osteoclasts are inactive, the presence of abundant reversal lines and continued growth of these lesions suggests that this is not the case. This nevertheless remains to be confirmed by examining in greater detail the morphology of the transgenic osteoclasts and by measuring their resorptive activity *in vitro* (see also below). Moreover, the fact that some of the transgenic mice developed very large bone tumours also suggests that additional events are occurring following expression of the transgene, such as activation of genes which are important in the growth control of specific bone cell populations (Sunters *et al*, 1998; Sunters *et al*, 2000). While this is a possible scenario, we cannot exclude the possibility that the early lesions would have eventually expanded extracortically to resemble more the tumour lesions (e.g., Figure 3.2 B), and which would not have been observed because the mice were sacrificed at a relatively early age.

Expression analysis of other AP-1 family members in this study demonstrated that *fra*-1, a c-Fos responsive gene thought to be important for osteoclast differentiation and function, is up-regulated in the long bone and tumours of TRAP-c-*fos*LTR mice. Fra-1 has been shown to be the most potent osteoclastogenic Fos-family member protein *in vitro*, but unlike other cell types, transcriptional activation in osteoclasts requires c-Fos protein (Owens *et al*, 1999; Matsuo *et al*, 2000). Together c-Fos and Fra-1 mediate the osteoclastogenic stimulatory effect of RANKL/RANK signalling in osteoclasts (Matsuo *et al*, 2000). Furthermore, Fra-1 together with NFAT3 have been shown to stimulate the TRAP promoter *in vitro* (Matsuo *et al*, 2000). In TRAP-c-*fos*LTR mice Fra-1 may be further augmenting any effects of c-Fos, by additionally stimulating the exogenous TRAP promoter and hence increasing ectopic c-*fos* transgene expression. Thus, the c-Fos induction of *fra*-1 expression in TRAP-c-*fos*LTR mice could be increasing the differentiation potential of osteoclasts.

Assessing the possible causes of tumour formation in TRAP-c-*fos*LTR mice is very important, as the tumours resemble, to a certain extent, the remodelling osteosarcomas which develop in other c-Fos transgenic mice (H2-c-*fos*LTR; Grigoriadis

et al, 1993). In H2-c-*fos*LTR mice, chondroblastic osteosarcomas develop due to specific transformation of osteoblastic cells, and osteoblasts express high levels of the transgene. The fact that there are large numbers of osteoblasts (and in some cases chondrocytes) in TRAP-c-*fos*LTR lesions, a large proportion of which expressed c-Fos protein by immunocytochemical analysis (Figure 3.4) and c-Fos/pB15 transgene (Figure 3.5), raises several possible interpretations: First, since the c-Fos antibody used cannot distinguish between endogenous and exogenous c-Fos, it is possible that c-Fos expression in osteoblasts reflects endogenous c-Fos protein; efficient expression of the endogenous gene is confirmed by the Northern blot analyses. This may be possible since expression of endogenous c-Fos in osteoclasts is normally very low (Sunters *et al*, 1998) and the activation osteoblasts by osteoclasts during bone formation may indeed turn on endogenous c-Fos expression (Grigoriadis *et al*, 1993). Second, it is possible that high c-Fos levels in osteoblasts could be interpreted as inappropriate expression of the transgene from the TRAP promoter, as evidenced by non-specific transgene expression in osteoblasts, such that, there was 'leaky' expression of the TRAP promoter in osteoblasts leading to the observed tumour phenotype. Possible explanations for this scenario might be attributed to the 3' FBJ-LTR which was used in the construct. Although it has been demonstrated unequivocally that this fragment is necessary for efficient expression of c-Fos in bone tissue, it cannot be excluded that there exist sequences within this fragment which can override or alter the specificity of the selected promoter. The evidence that this could indeed occur in transgenic mice has previously been discussed (Grigoriadis *et al*, 1993; Wang *et al*, 1991).

Taken together, the skeletal abnormalities which develop in TRAP-c-*fos*LTR transgenic founder mice share some properties with Paget's disease, namely, an increase in the number of large, active osteoclasts which express high levels of c-Fos, increased remodelling activity, and cellular transformation and tumour formation. Other characteristics are also shared, for example, high Bcl-2 levels in osteoclasts (Haslam *et al*, 1997; Cody *et al*, 1997; Mee, 1999), were observed in TRAP-c-*fos*LTR mice. Furthermore, preliminary evidence suggests that in the remodelling osteosarcomas in H2-c-*fos*LTR mice (Grigoriadis *et al*, 1993), high levels of Bcl-2 expression have also been observed in osteoclasts (E. El-Emir and A.E. Grigoriadis, unpublished observations). The increased expression of Bcl-2 may account for the

increased potential for cellular transformation and tumourigenesis observed in these mice.

The results from this study are based solely on founder animals, as it has been difficult to obtain further families of transgenic mice. It is very clear that independent families of transgenic mice must be generated to determine unequivocally parameters such as the time of onset of transgene expression, the time of onset of microscopic phenotypic changes, the target cells for transformation in these mice, etc. Furthermore, establishing lines of TRAP-c-*fos*LTR transgenic mice will also provide a useful tool for investigating osteoclast activity *in vitro*, for example by investigating the osteoclastogenic capacity or altered gene expression of haematopoietic spleen precursors, or by culturing cells derived from tumours in the presence of RANKL (Lacy *et al*, 1998; Kong *et al*, 1999; Yasuda *et al*, 1998), which is essential for osteoclast differentiation. It is also likely that immortalised osteoclastic cell lines can be established from these lesions, as has been shown for other oncogenes targeted to osteoclasts (Hentunen *et al*, 1997). Finally, although TRAP-c-*fos*LTR transgenic mice cannot be considered as definitive models for Paget's disease, their usefulness will become evident by investigating whether the increased osteoclast activity and remodelling defects can be inhibited using anti-resorptive compounds such as bisphosphonates, either in a preventative way, or to treat already formed skeletal lesions. It is becoming very clear that the causes of Paget's disease will be multifactorial. The generation of TRAP-c-*fos*LTR mice will address only one component of the observed abnormalities in Paget's disease, namely, the up-regulation of c-Fos, and will provide a well-defined, readily manipulatable system to test the consequences of altered c-Fos expression in osteoclasts.

4. Effects of ectopic c-Fos expression on osteoblast proliferation and differentiation *in vitro*

4.1 Introduction

c-Fos and AP-1 have been shown to be important in regulating the activity and differentiation of different bone cell types (as detailed in Chapter 1 and demonstrated in Chapter 3). In addition to the effects of c-Fos on osteoclasts, gain-of-function studies have shown that c-Fos specifically transforms osteoblasts (Grigoriadis *et al*, 1995; Jochum *et al*, 2001). However, the role of c-Fos in the differentiation, activity and effect on c-Fos target genes in osteoblasts have not been fully investigated. Previous work in our laboratory has shown that cyclin D1 expression is specifically elevated in the pre-malignant osteoblasts and chondrocytes of H2-c-*fos*LTR mice and not in other transgene expressing tissues (Sunters *et al*, 1998), suggesting that cell cycle regulation may be one target of c-Fos in osteoblast and chondrocyte transformation.

Furthermore, using a tetracycline-inducible c-Fos expression vector, pJMF2-c-*fos*, Sunters *et al* (2000) generated stable clones of the osteoblastic cell line, MC3T3-E1 (AT9.2 cells). In this system, exogenous c-Fos resulted in a reduction in cell cycle time and premature G_1-S phase transition via deregulated cyclin E/A-CDK2 dependent mechanism, suggesting that deregulated c-Fos expression can alter osteoblast growth, which may contribute to malignant transformation. However, the effects of c-Fos overexpression on the osteoblast phenotype have not been fully investigated. AP-1 sites are found in the promoters of many bone specific genes, such as alkaline phosphatase, osteocalcin, collagen I and MMP-13 (Schule *et al*, 1990; Schonthal *et al*, 1988; Matsuura *et al*, 1990; Owen *et al*, 1990), suggesting a potential role for c-Fos in osteoblast differentiation.

In this chapter, investigations into the effects of ectopic c-Fos expression on the osteoblast phenotype have been described. Additional clones of MC3T3-E1 cells stably transfected with the pJMF2-c-*fos* vector were generated and the effects of exogenous c-Fos on osteoblast proliferation and differentiation were investigated.

4.2 Generation of stable cell lines of MC3T3-E1 cells which overexpress c-Fos

The osteoblast-like cell line MC3T3-E1 was transfected with pJMF2-c-*fos* (Thomas *et al*, 2000), a tetracycline (Tc)-regulatable construct that results in expression of

exogenous c-*fos* in the absence of Tc (Figure 4.1). After transfection, cells were selected in medium supplemented with 300μg/ml Geneticin (G418) and 10μg/ml Tc, and isolated by ring cloning (as descibed in section 2.6.5).

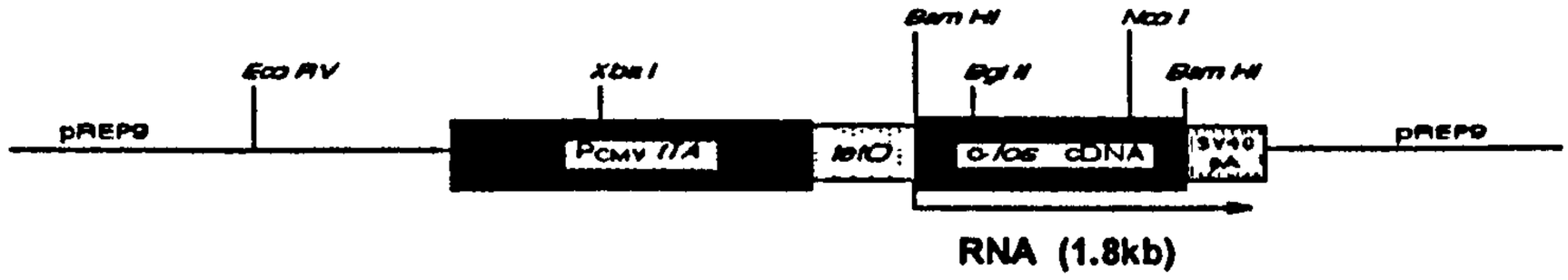


Figure 4.1 - The DNA construct pJMF2-c-*fos*. Regulation of c-*fos* expression is provided by constitutive expression of the Tc-repressed transactivator from a CMV promoter (P_{CMV} *tTA*). In the absence of Tc, the tTA protein binds to the Tc operator sequence ligated to a minimal human CMV promoter (*tetO*), directing the expression of c-*fos*. Transcription terminates at the SV40 poly A site (SV40 pA) of the expression vector pREP9 (Thomas *et al* 2000). The pJMF2-*luc* construct is identical to pJMF2-c-*fos* with the c-*fos* cDNA being replaced with a Firefly luciferase gene (*luc*).

Of the 24 G418-resistant clones isolated, 15 were examined for exogenous c-*fos* expression by Northern blot analysis using poly $(A)^+$ RNA isolated from cells grown in the absence and presence of Tc (Figure 4.2). At least 6 strongly expressing, positive clones (KT1.5, 1.20, 1.14, 1.22, 1.2, 1.3) and two weakly positive clones (KT1.7, 1.9) were identified. Four of these (KT1.5, 1.20, 1.14, 1.22) were selected for further screening of the c-Fos protein by Western blot analysis (Figure 4.3). Clones KT1.5 and KT1.20 were shown to have the strongest levels of mRNA transcripts for exogenous c-Fos and c-Fos protein when cells were grown in the absence of Tc. Protein levels were reduced dramatically in the presence of 1μg/ml Tc and completely absent at 10μg/ml Tc (Figure 4.3). Clone KT1.5 was selected for further studies because it showed the best dose-dependent regulation in response to Tc. Clones KT1.14 and KT1.22 showed very little c-Fos protein when grown in the

absence of Tc, and were not studied further. KT1.5 and KT1.20 were initially induced to undergo differentiation in the presence of BMP-2 and KT1.5 was chosen from this experiment for subsequent differentiation experiments since it showed a greater response to BMP-2-induced alkaline phosphatase activity (see below).

In addition, stable clones of MC3T3-E1 cells were generated following transfection with pJMF2-*luc* (e.g., KT2.1) which encodes the luciferase (*luc*) gene instead of c-*fos*. Seven stable G418-resistant clones were grown in the presence or absence of Tc and the extracts were assessed for luciferase activity (see Table 4.1). Clone KT2.1 was used for further studies as a control for transfection and selection, as well as any effects that the withdrawal of Tc may have on the cells.

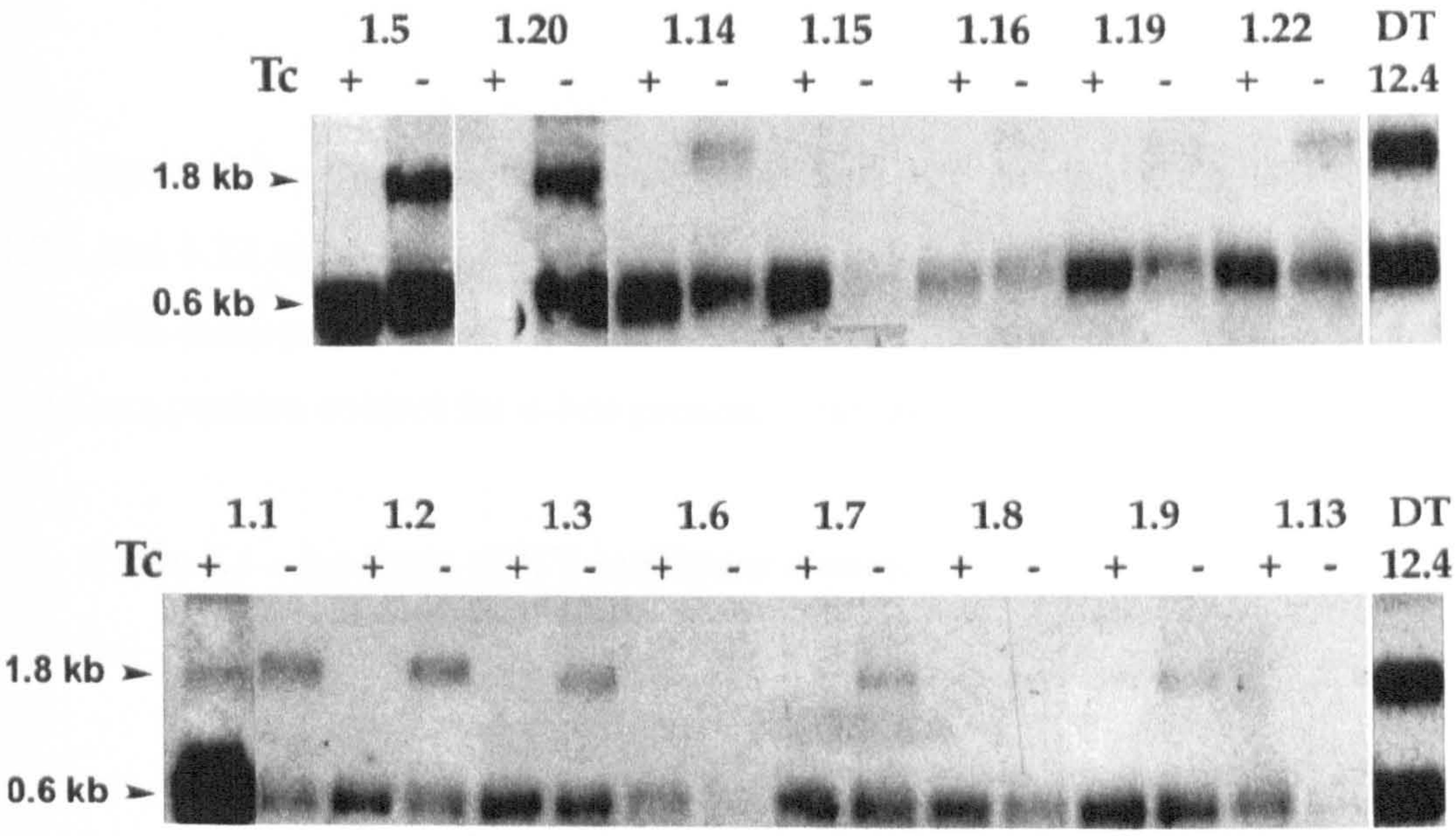


Figure 4.2 – Analysis of exogenous c-*fos* expression in KT clones. Northern blot analysis of poly (A)+ RNA from KT clones cultured in the presence and absence of Tc (10μg/ml) for 3-4 days. The membrane was hybridised with a ^{32}P-labelled v-*fos* probe which detects the exogenous (1.8 kb) c-*fos* transcript. The endogenous *fox* gene (0.6 kb) was used as a control for RNA loading. RNA extracted from pJMF2-c-*fos* transfected ATDC5 cells (DT12.4; Thomas *et al*, 2000) was used as a positive control.

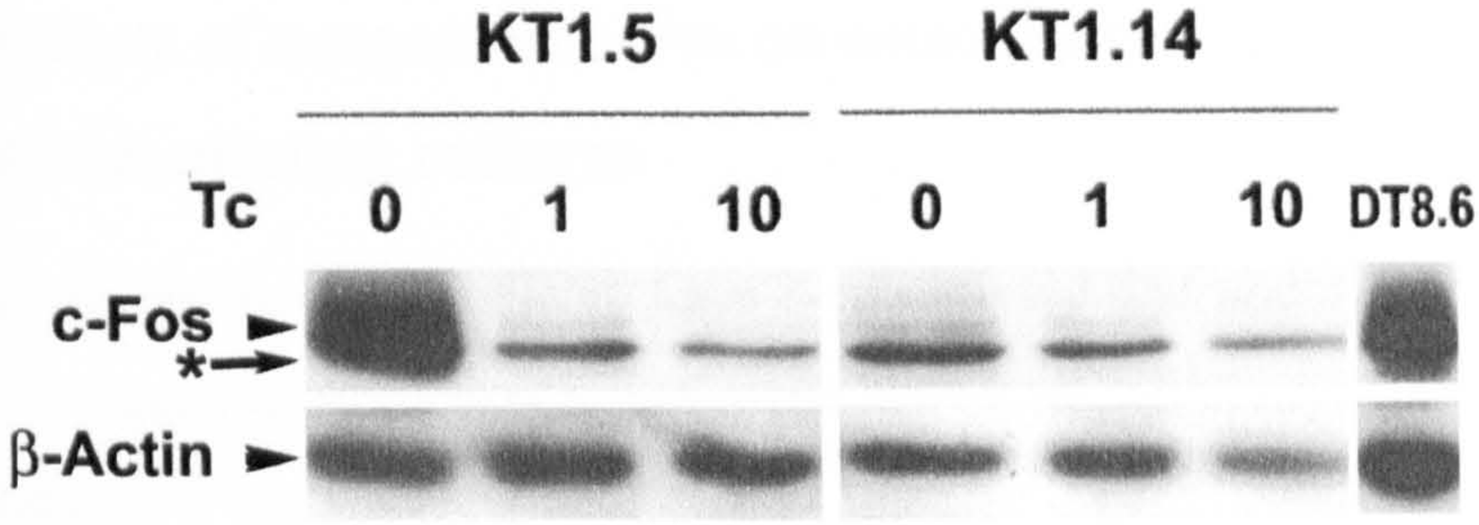


Figure 4.3 – Western blot analysis of four KT clones. Cells (KT1.5, 1.14, 1.20, and 1.22 cells) were cultured for 4 days before Western blot analysis (as described in section 2.9). A chondrogenic ATDC5 c-Fos expressing clone, DT8.6, was used as a positive control for c-Fos protein. */arrow – non-specific band.

Table 4.1 - Analysis of KT luciferase clones.

	Luciferase activity (Light intensity / μg protein)		
Clone	+Tc	-Tc	Fold-induction
KT2.1	0.00	0.27	-
KT2.2	0.06	0.14	2.3
KT2.3	0.06	0.05	0.8
KT2.4	0.1	34.88	348.8
KT2.5	1.99	0.14	0.1
KT2.6	0.07	0.12	1.7
KT2.7	0.08	0.01	0.1
AT7.8	503.50	4882.00	9.7

Luciferase activity of cell lysate from KT *luc* clones grown ± Tc (10μg/ml). Luciferase activity of lysates was measured using Promega Firefly Luciferase Assay System protocol (see section 2.6.6.4). Lysate from pJMF2-*luc* transfected MC3T3-E1 cells (AT7.8) was used as a positive control.

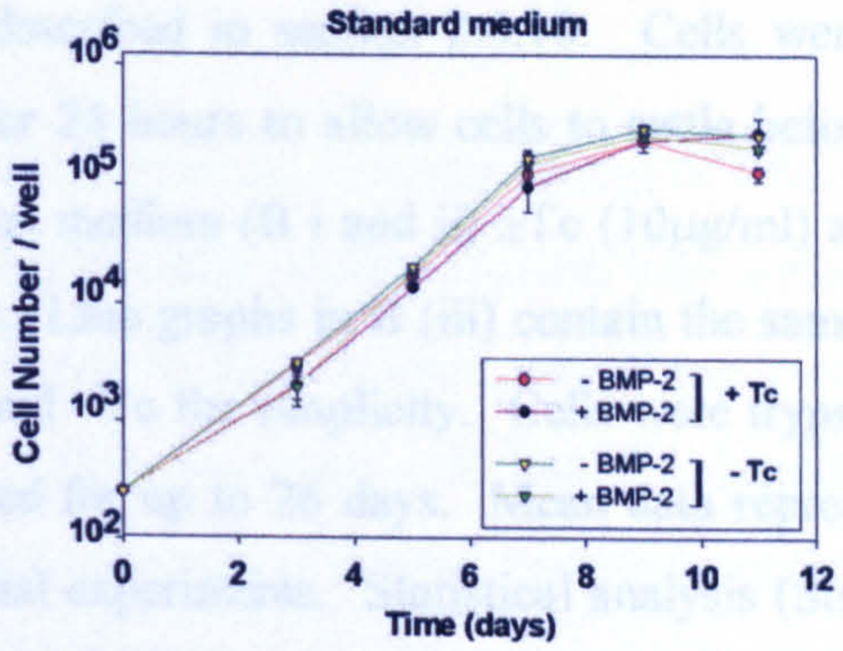
4.3 Effects of exogenous c-Fos on osteoblast proliferation in differentiating cultures

Previous studies in our laboratory have demonstrated that under normal serum conditions, exogenous c-Fos expression alone shows no effect on osteoblast proliferation (Sunters *et al*, 2000). Parental wild type MC3T3-E1 cells can be induced to undergo osteoblast differentiation in the presence of β-glycerophosphate and ascorbic acid, and also BMP-2 (as described in method section 2.6.7). When KT1.5 cells were induced to undergo differentiation in these conditions and in the presence of Tc, a greater number of cells appeared to be present in BMP-2 treated cultures when viewed by phase contrast light microscopy. This stimulation of proliferation was apparently further augmented by exogenous c-Fos expression (data not shown). To investigate in detail the role of c-Fos in osteoblast proliferation, KT1.5 cells were assessed by growth curve analysis. Under standard culture conditions in 10% serum, the withdrawal of Tc induced no changes in proliferation either in the presence or absence of BMP-2 (Figure 4.4 A). However, under differentiation conditions and in the absence of Tc there was evidence of increased proliferation of KT1.5 cells at early time points (days 10 to 15), corresponding to the log phase of growth and the onset of multilayering (Figure 4.4 B). By day 16, of which time there is abundant multilayering and extracellular matrix deposition, cell proliferation in the presence and absence of Tc was eventually the same (Figure 4.4 B).

To confirm that the stimulation of proliferation was due to the effects of exogenous c-Fos and not due to the withdrawal of Tc from the culture medium, growth curve analysis was performed for the parental cells MC3T3-E1 and KT2.1 (luc) cells under differentiation conditions. MC3T3-E1 cells were initially cultured for 3-4 passages in the presence of Tc (10μg/ml) and then cultured under differentiation conditions for up to 8 days in Tc (0, 1, 10μg/ml). Cell numbers were counted at varying time points during the log phase of growth. MC3T3-E1 cells appeared to exhibit no change in proliferation in the presence or absence of Tc (Figure 4.5 A). However, an inhibition of KT2.1 cell proliferation was observed in the presence of 10μg/ml Tc (Figure 4.5 B). This indicates that in the transfected clones, Tc has a slight inhibitory effect on cell proliferation, and that the apparent increase in KT1.5 cell number following Tc withdrawal may not be due solely to increased exogenous

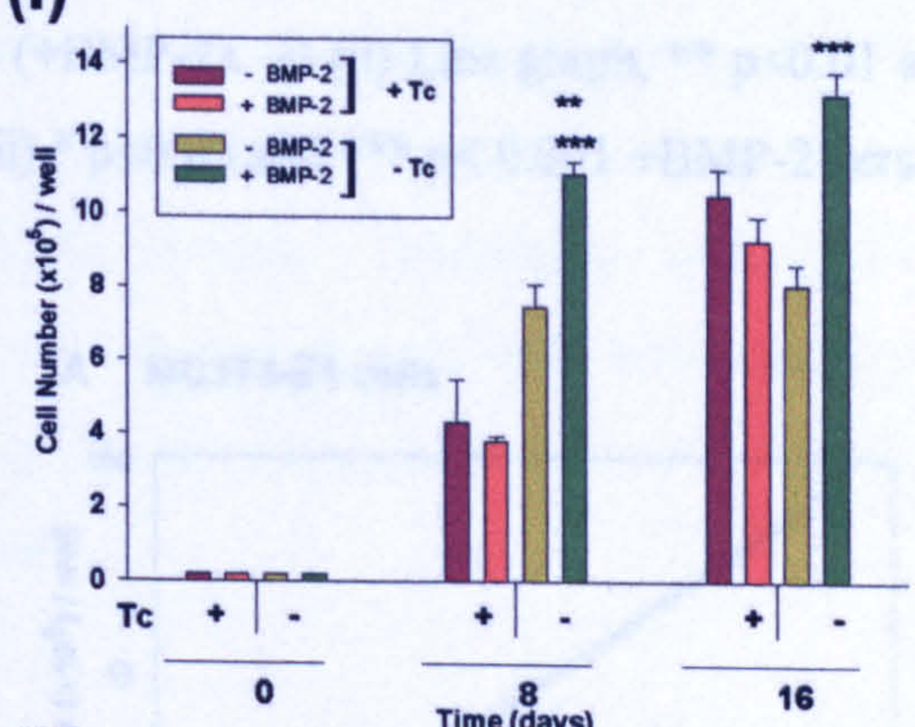
c-*fos* expression. Consistent with this possibility, Sunters *et al* (2000) found that in 10% serum, AT9.2 cells showed no changes in proliferation either in the presence and absence of Tc. Not withstanding the possible effect of Tc on KT1.5 cell proliferation, BMP-2 was found to significantly increase cell number in the presence, but not absence, of exogenous c-*fos* expression, during the late exponential growth of the cultures. Based on these findings, further experiments to determine the effects of exogenous c-Fos expression on the differentiation of KT1.5 cells, were conducted in the presence of Tc at 1μg/ml.

A Standard medium

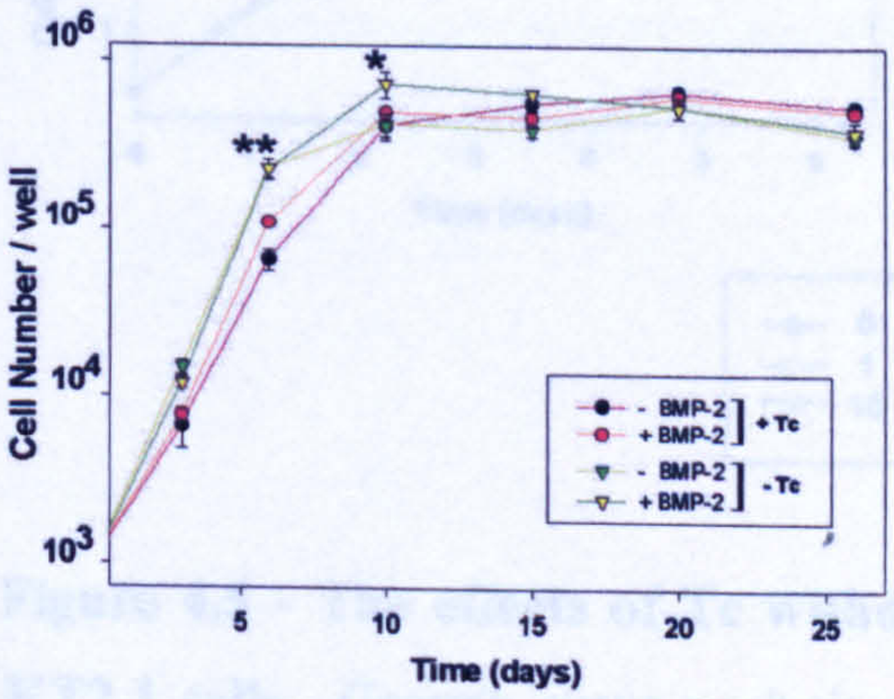


B Differentiation medium

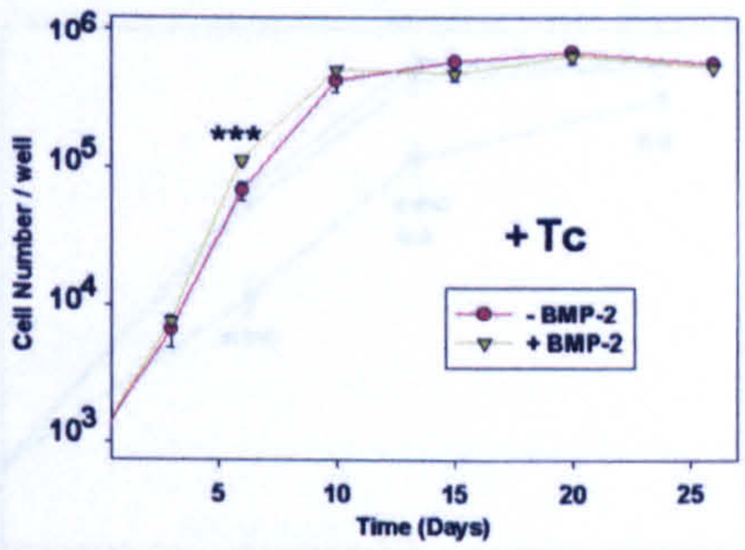
(i)



(ii)



(iii)



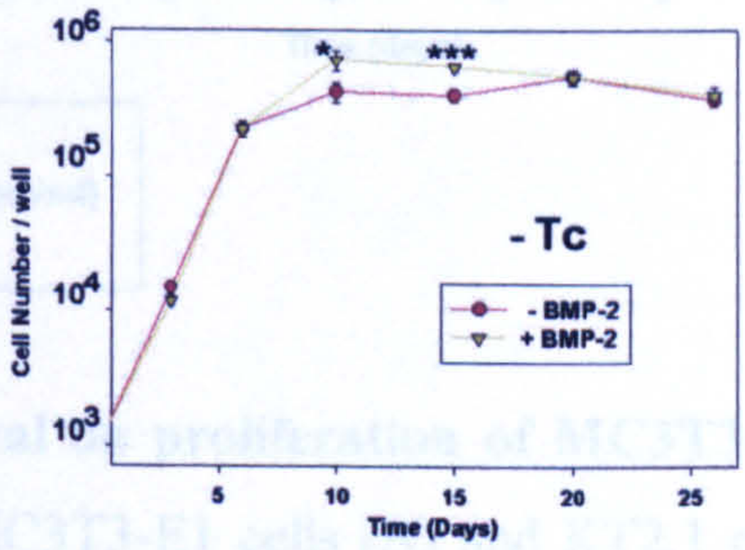


Figure 4.4 - The effects of exogenous c-Fos on proliferation of KT1.5 cells (figure on previous page). Growth curve analysis of cells grown in (A) standard medium and (B) differentiation medium as described in section 2.6.10. Cells were cultured overnight in standard medium with Tc for 24 hours to allow cells to settle before treatment in standard medium (A) or differentiation medium (B i and ii) ±Tc (10μg/ml) and ±BMP-2 (100ng/ml) for the indicated time points. Line graphs in B (iii) contain the same data from (ii) but data has been divided into +Tc and –Tc for simplicity. Cells were trypsinised and cell numbers counted at the times indicated for up to 26 days. Mean data represents cell counts from 3 separate wells from individual experiments. Statistical analysis (Student's t-test): B (i) Bar graph, day 8, ** $p<0.01$ for +BMP-2 versus –BMP-2 (–Tc) and *** $p< 0.001$ for –Tc versus +Tc (+BMP-2); day 16 *** $p=0.001$ for +BMP-2 versus –BMP-2 (–Tc) and –Tc versus +Tc (+BMP-2). B (ii) Line graph, ** $p<0.01$ and * $p<0.05$ for +Tc versus -Tc (+BMP-2). B (iii) * $p<0.05$ and *** $p< 0.001$ +BMP-2 versus –BMP-2.

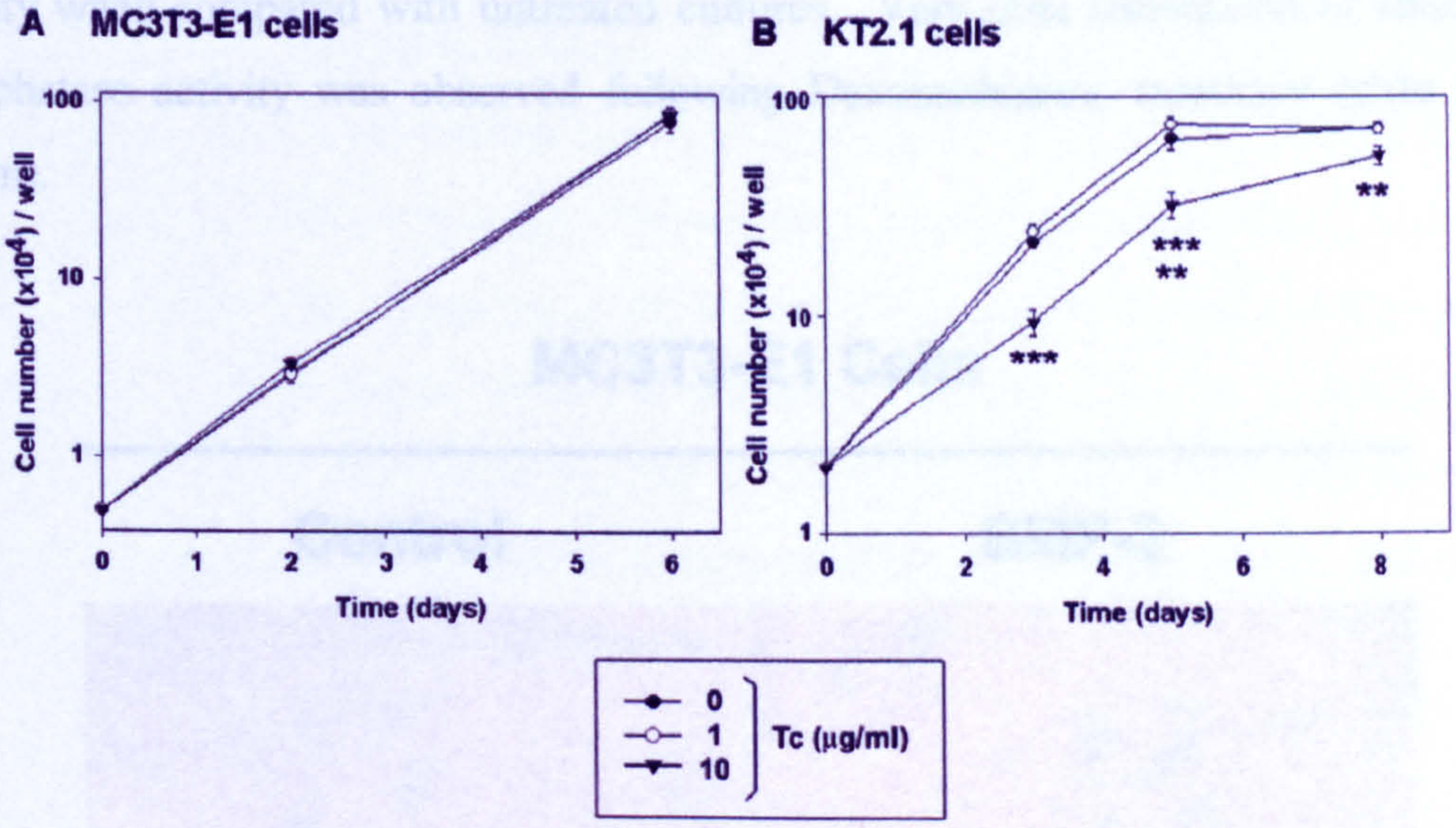


Figure 4.5 - The effects of Tc withdrawal on proliferation of MC3T3-E1 and KT2.1 cells. Growth curve analysis of MC3T3-E1 cells (A) and KT2.1 cells (B) grown in differentiation medium as described in section 2.6.10. Cells were cultured in standard medium for 24 hours before treatment in differentiation medium ± Tc (0, 1, 10μg/ml) as indicated. Cell numbers were counted at the times indicated for up to 8 days. Mean data represents cell counts from 3 separate wells from individual experiments. *** $p<0.001$ 10μg/ml Tc versus 0 and 1μg/ml Tc (day 3); *** $p<0.001$ 10μg/ml Tc versus 1μg/ml Tc and ** $p<0.01$ 10μg/ml Tc versus 0 μg/ml Tc (day 5); ** $p<0.01$ 10μg/ml Tc versus 0 and 1μg/ml Tc (day 8).

4.4 Effects of BMP-2 on differentiation of MC3T3-E1 and KT1.5 cells

The effects of exogenous c-Fos on the responsiveness of osteoblastic cells to factors known to be important for osteoblast differentiation was investigated next. Parental MC3T3-E1 cells were cultured in the presence and absence of BMP-2 to assess the differentiation potential of this particular cell line (as detailed in methods section 2.6.7). In untreated cultures there was limited expression of alkaline phosphatase as measured by histochemistry (Figure 4.6). Structures resembling bone nodules were not seen. Treatment with BMP-2 increased the number of alkaline phosphatase positive foci and their staining intensity (Figure 4.6). In addition, MC3T3-E1 cells were differentiated in the presence of either Dexamethasone (100nM) or 1,25-$(OH)_2D_3$ (10nM). Treatment with 1,25-$(OH)_2D_3$ increased alkaline phosphatase activity when compared with untreated cultures. Very little stimulation of alkaline phosphatase activity was observed following Dexamethasone treatment (data not shown).

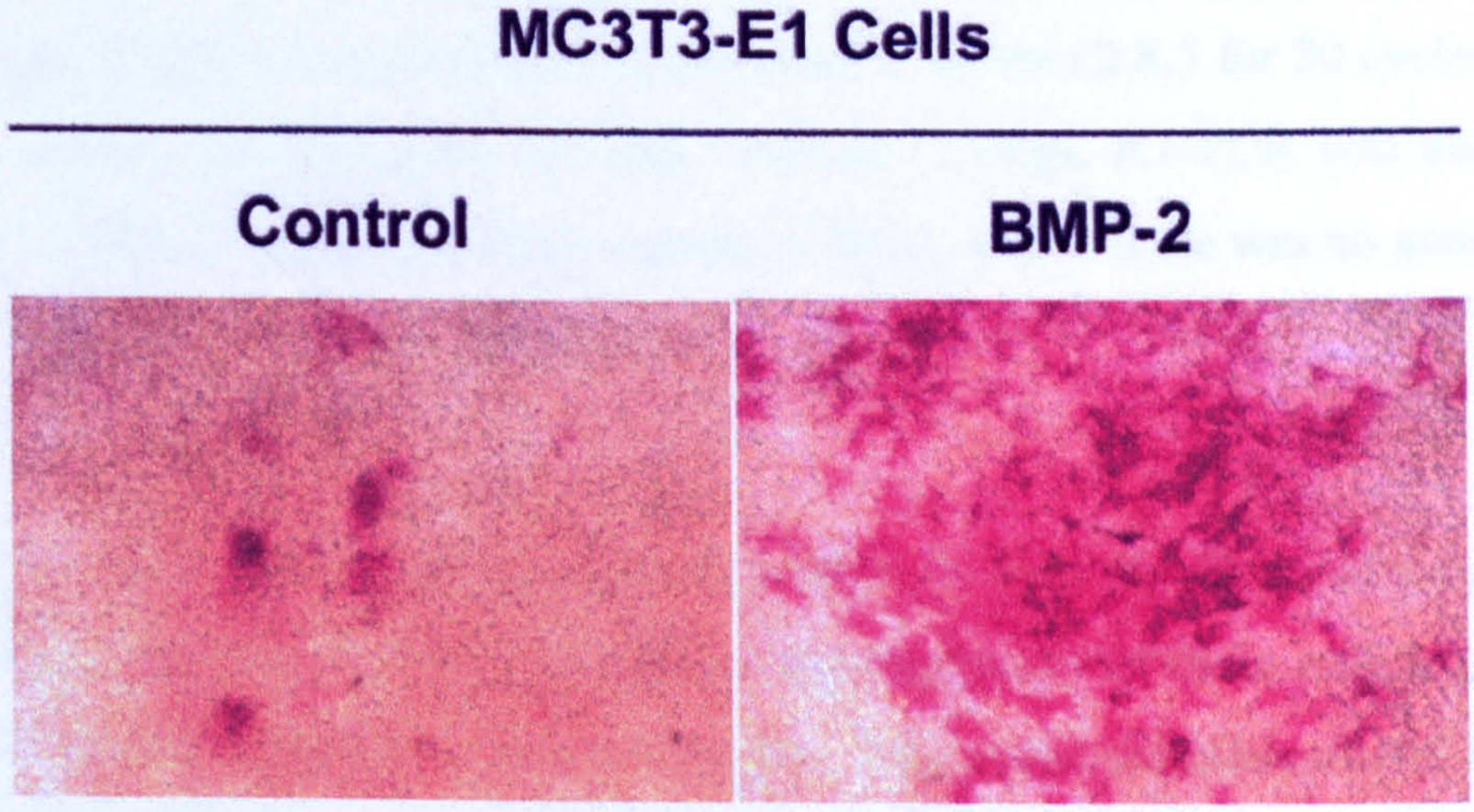


Figure 4.6 - Differentiation of MC3T3-E1 cells following treatment with BMP-2. Cells were cultured for 35 days ± BMP-2 (100ng/ml) under differentiation conditions (section 2.6.7) before cultures were fixed and stained for alkaline phosphatase activity (section 2.6.8).

In MC3T3-E1 cells, the expression of alkaline phosphatase increased with time during the phase of differentiation. Using semi-quantitative RT-PCR of total RNA extracted at days 1, 15 and 30 of differentiation, cells demonstrated a time-dependent increase in alkaline phosphatase mRNA expression over the time course of differentiation, which was stimulated further by BMP-2 treatment (Figure 4.7).

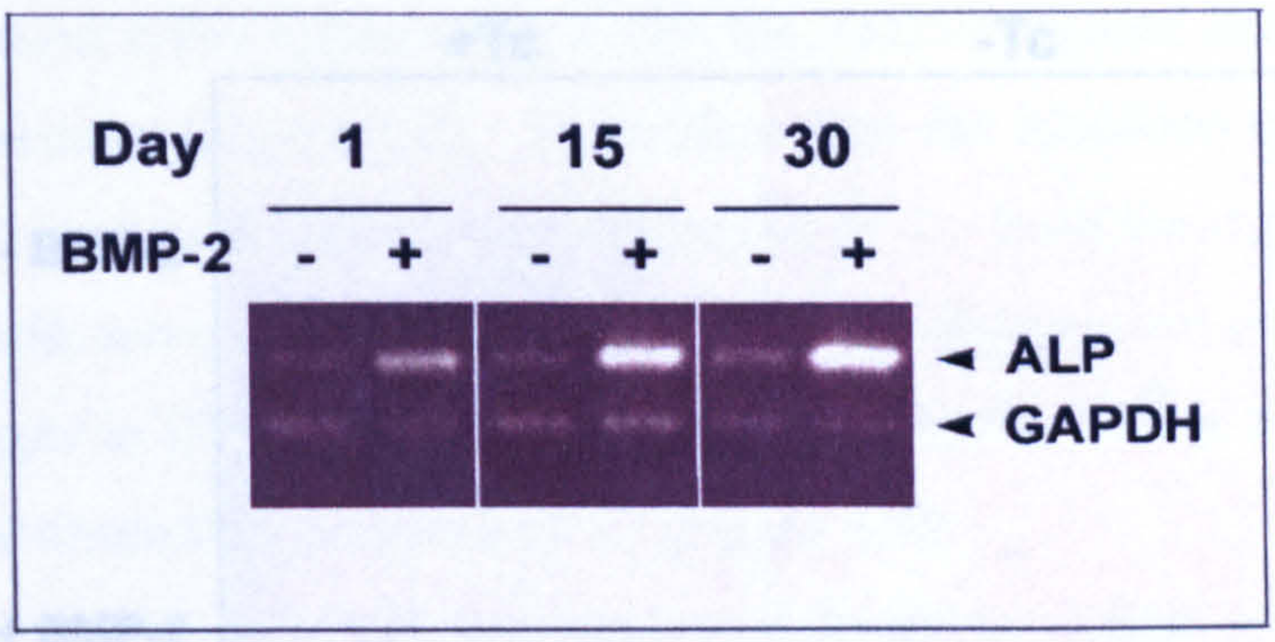


Figure 4.7 - RT-PCR analysis of basal and BMP-2 stimulated alkaline phosphatase (ALP) expression in MC3T3-E1 cells. Cells were cultured for 30 days ± BMP-2 (100ng/ml) under differentiation conditions as described in section 2.6.7. Total RNA was extracted at the indicated time points throughout the culture period, and RT-PCR was performed as described in section 2.8.5 for 30 cycles using primers specific for ALP (409 bp) and GAPDH (250bp). RT-PCR was also performed on samples containing water instead of RNA, where there was no generation of PCR products found (data not shown).

KT1.5 cells were also differentiated in culture to study the effects of exogenous c-Fos on the osteoblast phenotype and the responsiveness of these cells to growth factors following exogenous c-*fos* expression. The clonal selection of MC3T3-E1 cells following transfection with pJMF2-c-*fos* or pJMF2-*luc* resulted in a change in phenotype of the cells. Although KT1.5 cells grown in the presence of Tc remained capable of differentiating, they did so only in the presence of BMP-2. Cells that had been differentiated for 35 days in the presence of Tc and BMP-2 showed a small number of alkaline phosphatase positive cell foci (Figure 4.8). In the absence of Tc, that is, in the presence of exogenous c-Fos the number and intensity of staining of these foci was increased. AT9.2 cells, an additional c-Fos overexpressing MC3T3-

E1 clone generated by Dr. A. Sunters (Sunters *et al*, 2000), also showed increased numbers of alkaline phosphatase positive foci in the absence of Tc (Figure 4.8).

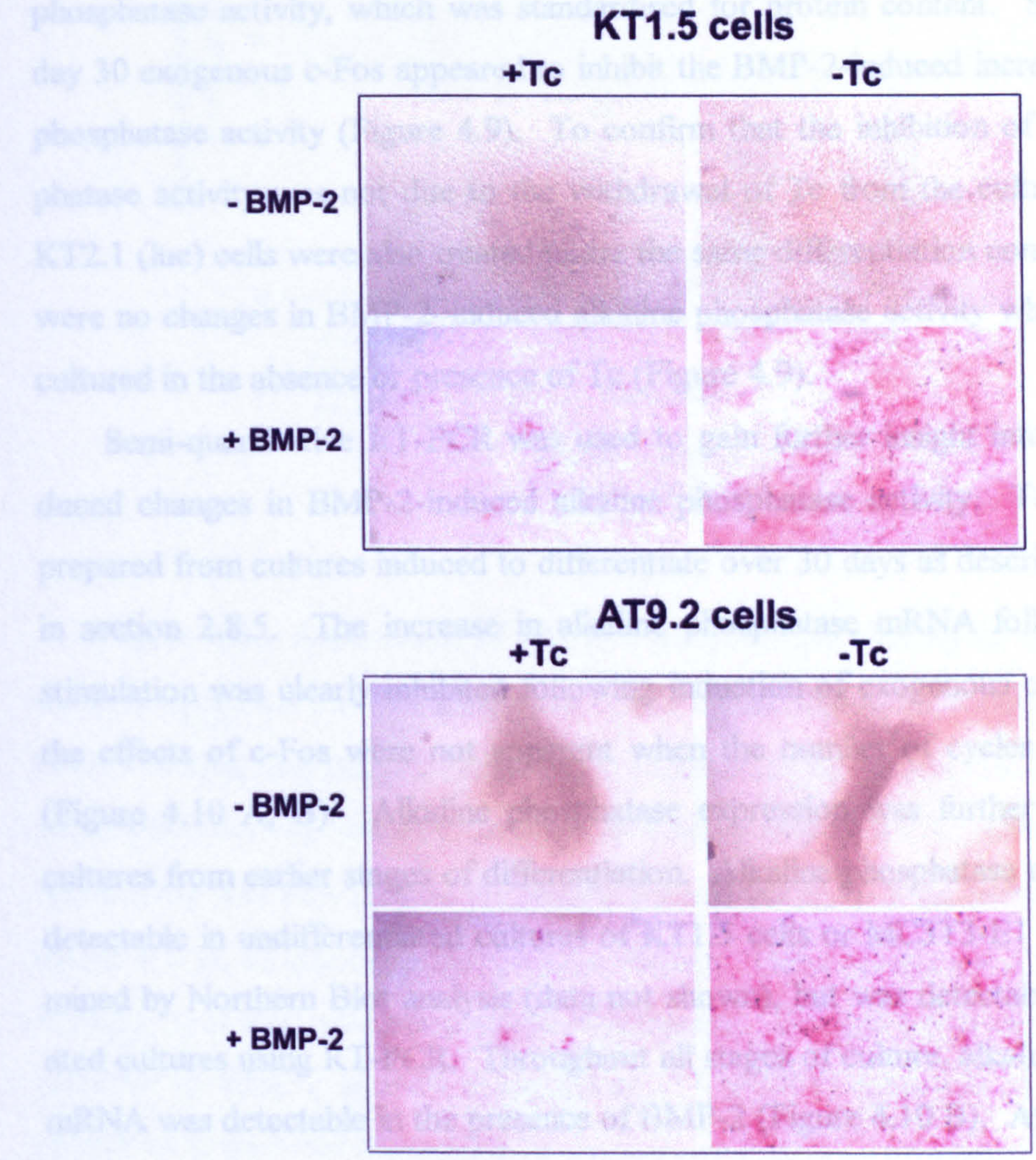


Figure 4.8 – Histochemical staining for alkaline phosphatase (ALP) activity following differentiation of KT1.5 and AT9.2 cells. KT1.5 cells were cultured for 35 days ± Tc (1μg/ml) and ± BMP-2 (100ng/ml) under differentiation conditions before cultures were fixed and stained for ALP (as detailed in section 2.6.8).

The effect of exogenous c-Fos on BMP-2-induced alkaline phosphatase activity in KT1.5 cells was further investigated using a quantitative measure of alkaline phosphatase activity and semi-quantitative RT-PCR. Cells were grown for 30 days in differentiation medium, in the presence and absence of exogenous c-Fos induction, and with or without BMP-2 treatment. Extracts were assessed for alkaline phosphatase activity, which was standardised for protein content. Surprisingly, at day 30 exogenous c-Fos appeared to inhibit the BMP-2-induced increase in alkaline phosphatase activity (Figure 4.9). To confirm that the inhibition of alkaline phosphatase activity was not due to the withdrawal of Tc from the culture conditions, KT2.1 (luc) cells were also treated under the same differentiation conditions. There were no changes in BMP-2–induced alkaline phosphatase activity when KT2.1 was cultured in the absence or presence of Tc (Figure 4.9).

Semi-quantitative RT-PCR was used to gain further insight into the c-Fos induced changes in BMP-2-induced alkaline phosphatase activity. Total RNA was prepared from cultures induced to differentiate over 30 days as described above and in section 2.8.5. The increase in alkaline phosphatase mRNA following BMP-2 stimulation was clearly inhibited following induction of exogenous c-Fos, although the effects of c-Fos were not apparent when the number of cycles was increased (Figure 4.10 A, B). Alkaline phosphatase expression was further determined in cultures from earlier stages of differentiation. Alkaline phosphatase mRNA was not detectable in undifferentiated cultures of KT1.5 cells or MC3T3-E1 cells, as determined by Northern Blot analysis (data not shown), but was detectable in differentiated cultures using RT-PCR. Throughout all stages of culture, alkaline phosphatase mRNA was detectable in the presence of BMP-2 (Figure 4.10 B). At days 5 and 16 alkaline phosphatase mRNA was detectable in BMP-2-treated cultures independant of exogenous c-Fos expression. At day 30, however, there was a marked reduction in the ability of BMP-2 to induce alkaline phosphatase mRNA in cultures maintained in the absence of Tc (Figure 4.10 C).

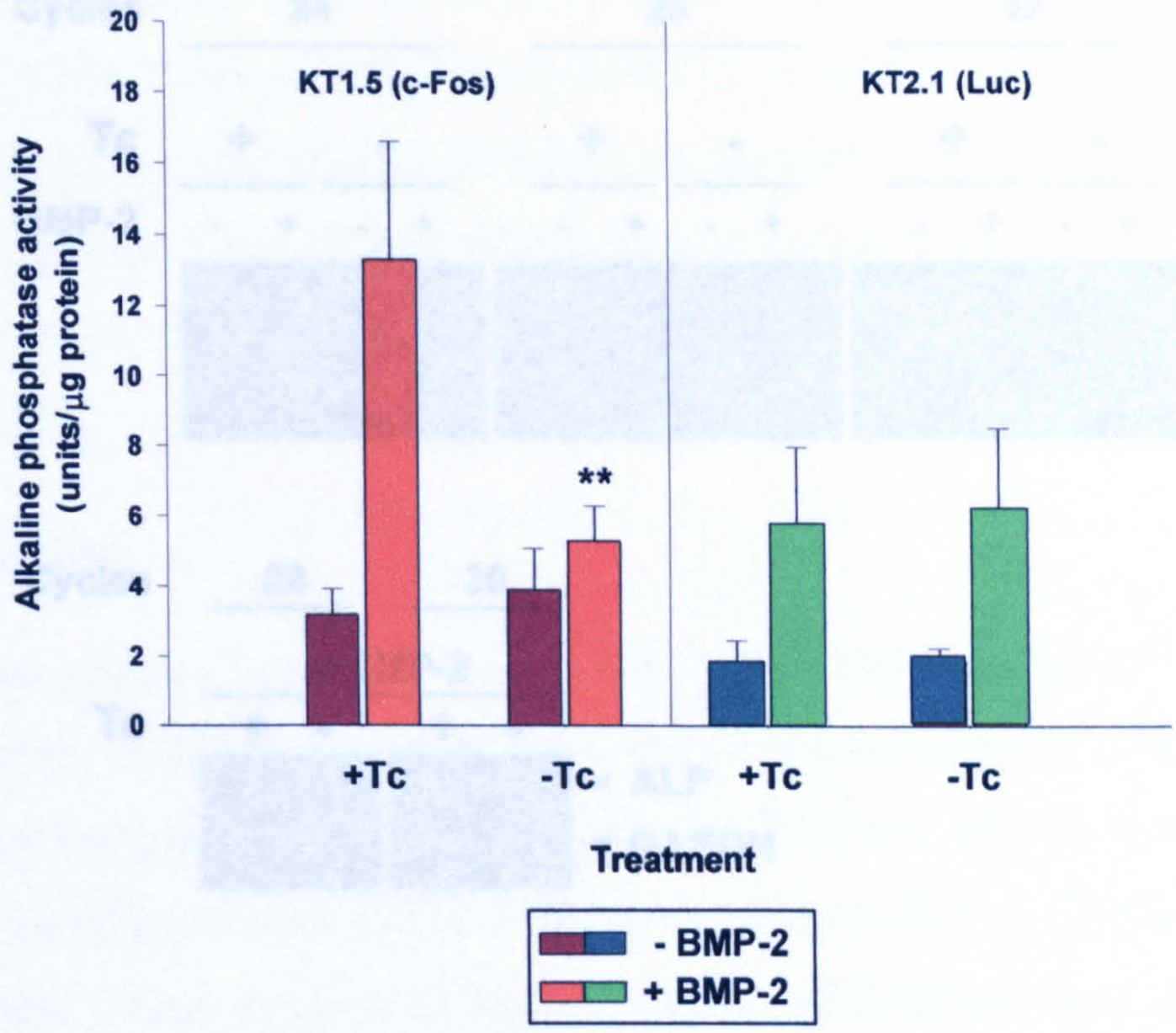


Figure 4.9 - Assessment of alkaline phosphatase activity in differentiated cultures of KT1.5 and KT2.1 clones. Cells were cultured for 30 days ± Tc and ± BMP-2 (100ng /ml) under differentiation conditions (see section 2.6.7) and alkaline phosphatase activity was determined as detailed in section 2.6.9. Alkaline phosphatase activity was standardised for protein content (units ALP/µg protein/well). The means ± S.D. of quadruple samples are shown. These results are a representative example of at least two independent experiments. Ectopic c-Fos expression in the presence of BMP-2 showed a significant (** $p<0.01$) reduction in ALP activity compared to no c-Fos expression.

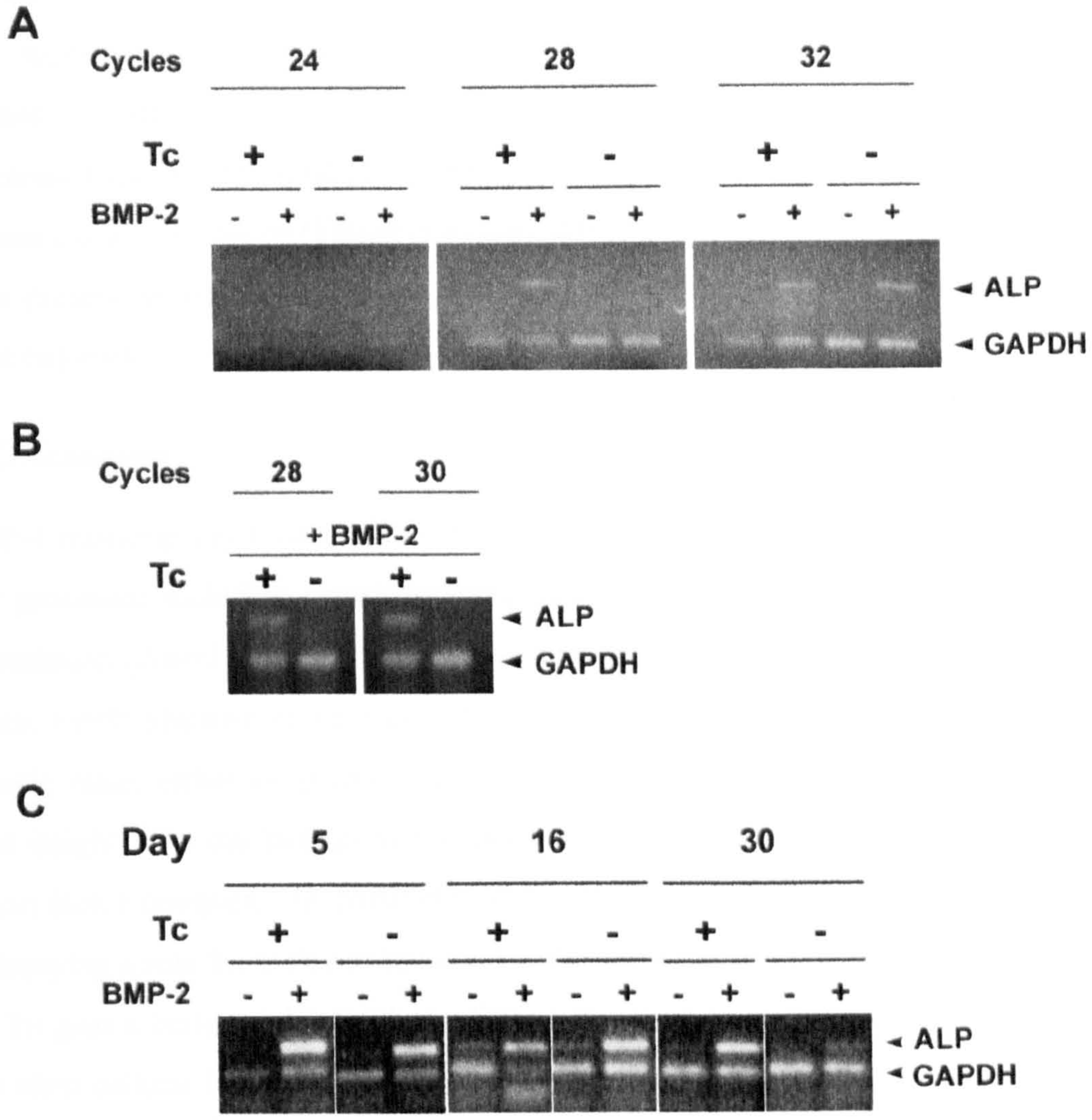


Figure 4.10 - RT-PCR analysis of basal and BMP-2 stimulated ALP expression in KT1.5 cells. Cells were cultured for 30 days ± Tc and ± BMP-2 (100ng/ml) under differentiation conditions as described in section 2.6.7. Total RNA was extracted at the indicated time points and RT-PCR was carried out using primers specific for ALP and GAPDH (see section 2.8.5). (A) Conditions were standardised for the final differentiation time point (day 30) by performing 24, 28, 32 PCR cycles and then repeated for 28 and 30 cycles (B). (C) PCR was performed for all indicated differentiation time points using 30 PCR cycles. PCR products are indicated – ALP (409 bp) and GAPDH (250bp). RT-PCR was also performed on samples containing water instead of RNA, where there was no generation of PCR products found (data not depicted).

From these results obtained by quantitative alkaline phosphatase assays and RT-PCR, it appears that following differentiation, ectopic c-Fos expression inhibits the expression of BMP-2-induced alkaline phosphatase at later stages of differentiation. The increased alkaline phosphatase staining that was reported in the cultures induced to express exogenous c-Fos (Figure 4.8) may have been a result of a greater number of cells present giving the appearance of larger numbers of alkaline phosphatase positive colonies.

4.5 Discussion

The AP-1 transcription factor, c-Fos, has been implicated in a large variety of biological processes including transformation, proliferation, apoptosis, and oncogenic transformation (Angel and Karin, 1991; Kovary and Bravo, 1991; Karin, 1997; Leibermann, 1998; Shaulian and Karin, 2001; Jochum *et al*, 2001). The generation of transgenic mice, either by gain-of-function or loss-of-function, have provided important insights into the biological functions of c-Fos component of the AP-1 transcription factor complex. In particular, c-Fos transgenic mice develop osteosarcomas, implying a role for c-Fos in bone cell differentiation, apoptosis and transformation. To gain a better understanding of the role of c-Fos in bone cell phenotype at the *in vitro* cellular level, the ectopic expression of c-Fos in MC3T3-E1 cells was studied. These cells represent a spontaneously immortalised (non-transformed) osteoblast cell line (Sudo *et al*, 1983), which behaves as an immature, committed osteoblast cell population that can undergo differentiation in response to intracellular and extracellular signs (see below).

MC3T3-E1 cells were transfected with a tetracycline (Tc)-regulatable c-Fos expression construct (pJMF2-c-*fos*), to generate stable clones, one of which (KT1.5) was selected for investigations into the effect of ectopic c-Fos expression on the osteoblast phenotype. Wild type MC3T3-E1 cells do not show any endogenous c-Fos expression either in the presence and absence of Tc under normal conditions. However, KT1:5 cells showed tight regulation by Tc of exogenous c-Fos expression. Furthermore, previous work by Sunters *et al* (2000) has shown that using this system, similar clones (e.g., AT9.2) were generated and the exogenous c-Fos protein that was produced was functional in terms of specific DNA binding and transactivation of reporter gene activity for AP-1 sequences.

4.5.1 Inducible overexpression of c-Fos leads to the inhibition of the osteoblast phenotype and an increase in proliferation

Ectopic c-Fos expression in a clonal cell line of MC3T3-E1 cells (KT1.5) was shown to down regulate the BMP-2 induced expression of alkaline phosphatase during the later stages of osteoblast differentiation, with no effect observed during the earlier stages.

Following clonal selection MC3T3-E1 cells, transfected with pJMF2-c-*fos*, showed a partial loss in their ability to differentiate. This was perhaps not surprising since it is well established that MC3T3-E1 cells lose their differentiation potential with increased passage number (Wang *et al*, 1999). Indeed, deriving stable sub-clones from a parental clonal population, involves many population doublings before a sufficient number of cells are obtained to perform reproducible molecular and bio-chemical experiments. Nevertheless, the cells were still considered osteoblastic since they retained their ability to differentiate in response to BMP-2, at least as represented by expression of alkaline phosphatase. This was confirmed by histo-chemical staining, in addition to using RT-PCR and biochemical techniques.

In the parental MC3T3-E1 cells, the expression of alkaline phosphatase in-creased with time in culture, and BMP-2 further stimulated its activity, whereas KT1.5 cells grown in the presence of Tc were essentially dependent upon BMP-2 for expression of alkaline phosphatase. Interestingly, induction of exogenous c-Fos following Tc withdrawal in long-term KT1.5 cultures caused a marked reduction in BMP-2-dependent alkaline phosphatase mRNA expression and activity, compared to cells grown without c-Fos induction. These data suggest that one of the possible functions of c-Fos is to inhibit BMP-2-induced osteoblast differentiation. This is consistent with preliminary data from our laboratory, where transient transfection of primary mouse calvarial osteoblasts with a c-*fos* expression vector, caused an inhibi-tion in the number of bone nodules formed and a concomitant decrease in alkaline phosphatase staining (Dr A. Sunters, personal communication). However, the re-sults using KT1.5 cells were in apparent contradiction with the histochemical stain-ing for alkaline phosphatase where exogenous c-Fos appeared to increase BMP-2 induced alkaline phosphatase activity after 30 days in culture. This potential dis-crepancy is now believed to be due to the presence of a higher cell number in the

BMP-2/c-Fos cultures resulting in an overall increased staining pattern for alkaline phosphatase.

Qualitative observations of the cells through phase contrast microscopy consistently showed an apparently greater number of cells in BMP-2 treated cultures following ectopic c-Fos expression compared to control cells. Preliminary growth curve analysis during the earlier differentiation phase of KT1.5 cells confirmed a small but significantly higher number of cells cultured in the presence of BMP-2 and this was further augmented by exogenous c-*fos* expression. However, it was difficult to document accurately the increases in cell number at later stages, due to the difficulties in obtaining efficient single-cell suspensions in ascorbic acid-containing cultures where there is abundant extracellular matrix. Thus, further experiments using alternative indicators of proliferation, such as Bromo-uridine incorporation, tritiated thymidine incorporation or DNA content will verify the effects of Tc withdrawal and BMP-2 on proliferation of KT1.5 cells. That c-Fos and AP-1 are important for cell proliferation has been shown previously. As described in sections 1.6.2 and 1.6.4, cyclin D1 is an AP-1 target gene, and inhibition of c-Fos using neutralising antibodies attenuates cellular responsiveness to mitogens in quiescent cells (Kovary and Bravo, 1991). However, c-Fos is clearly not essential for cell proliferation since c-Fos knockout cells proliferate normally (Field *et al*, 1992; Brusselbach *et al*, 1995; Shaulian and Karin, 2001; Jochum *et al*, 2001). This suggests functional redundancy amongst other c-Fos family members, and likely candidates include FosB, since c-Fos/FosB double knockout mice are ~30% smaller than wild-type littermates, and double knockout fibroblasts exhibit impaired activation of cyclin D1 following serum stimulation (Brown *et al*, 1998). That exogenous c-Fos may give the cells a proliferative advantage in response, for example, to addition of growth factors including BMP-2, is consistent with recent observations in our laboratory in which an additional independent MC3T3-E1 sub-clone, AT9.2, that also harbours a Tc-regulatable c-*fos* gene, exhibits accelerated S-phase entry in response to serum mitogens (Sunters *et al*, 2000; see also Introduction, section 1.6.4.).

The effects of c-Fos overexpression on differentiation and development vary depending on the cellular background. Previous *in vitro* studies have shown that c-Fos and other members of the AP-1 complex are differentially regulated during osteoblast differentiation (McCabe *et al*, 1995; Banerjee *et al*, 1996). During the pro-

liferative stage of the rat calvarial osteoblast life cycle, the levels of all Fos and Jun proteins are high, but gradually decline during the period of extracellular matrix production and mineralisation. In fully differentiated osteoblasts Fra-2 and JunD are the major proteins in the AP-1 complex (McCabe *et al*, 1995; Owen *et al,* 1990). This implies that c-Fos plays a critical role in osteoprogenitor proliferation and it has been hypothesised that enforced expression of Fos/Jun family members during proliferation and subsequent developmental stages maintains cells in a proliferative state and inhibits further maturation which is necessary for matrix mineralisation (Lian *et al*, 1991; Owen *et al,* 1990). To a certain extent, the results of *in vivo* gain-of-function studies are consistent with this "phenotype suppression hypothesis": High c-Fos expression does not affect the differentiation potential of embryonic stem (ES) cells, since ES cells overexpressing c-Fos efficiently contribute to the development of chimeric mice (Wang *et al*, 1991). In contrast, ectopic c-Fos expression inhibits differentiation of chondrocytes (Thomas *et al*, 2000) and chimeric mice obtained from c-Fos overexpressing ES cells develop chondrosarcomas (Wang *et al*, 1991). Moreover, c-Fos overexpression in H2-c-*fos*LTR mice results in transformation of osteoblasts leading to osteosarcomas, and it has been postulated that c-Fos immortalises a sub-population of osteoprogenitor cells which renders them susceptible to further transformation (Grigoriadis *et al*, 1993). In contrast, while high levels of c-Fos can perturb osteoblast differentiation and cause transformation, osteoblasts can differentiate normally in the absence of c-Fos, as shown in the c-Fos knockout mice (Wang *et al*, 1992; Johnson *et al*, 1992; Grigoriadis *et al*, 1994), possibly as a result of the compensatory actions of related family members.

That c-Fos may be involved in osteoblast differentiation and function is also implied by the fact that known regulators of osteoblast differentiation and proliferation such as, TGF-β, PTH, and 1,25-$(OH)_2D_3$, induce the expression of c-*fos* (Machwate *et al*, 1995; Palcy *et al*, 2000; Pearman *et al*, 1996; Kano *et al*, 1994; Clohisy *et al*, 1992; Candeliere *et al*, 1991). Interestingly, recent preliminary data from the 23rd annual ASBMR meeting (2001) have shown altered osteoblastic responsiveness to PTH treatment in osteoblasts derived from c-Fos knockout mice (Demiralp *et al*, 2001). Again, suggesting that c-Fos may function as an important mediator of growth factor signalling in osteoblasts. In this regard, the results in this chapter that exogenous c-Fos affects the BMP-2 signalling pathway is significant. The effects of

BMP-2 on osteoblast differentiation have been studied intensively over the past decade. Treatment of osteoblasts with BMP-2 is known to stimulate alkaline phosphatase activity and collagen synthesis, an effect which is enhanced in the presence of oestrogen, dexamethasone, or 1,25-$(OH)_2D_3$ (Takuwa *et al*, 1991). The actions of BMPs on osteoblast proliferation depend on the cell type (see review in Wozney, 1993). Generally, treatment of osteoprogenitor cells with BMPs leads to increased cell proliferation. In MC3T3-E1 cells, transient treatment with BMP-2 alone has been shown to stimulate alkaline phosphatase levels but not to affect proliferation (Takuwa *et al*, 1991). It is also well-established that BMP-2 treatment of non-osteogenic precursor cells (e.g., 10T1/2) can induce them to differentiate into an osteoblast phenotype. It is not yet known how the BMP-2 pathway and c-Fos/AP-1 interact in KT1.5 cells, although in 10T1/2 cells, the BMP-2 effects have been postulated to be mediated by the ERK/MAPK signalling pathway (Lou *et al*, 2000). The ERK/MAPK cascade is the most important mediator of increased expresion of c-*fos* and c-*jun* family members in response to growth factor treatment (Angel *et al*, 1988). JunB, together with c-Fos, is thought to play a major role in BMP-2 signalling in osteoblasts (Palcy *et al*, 2000). Thus it would be tempting to speculate that in KT1.5 cells, BMP-2 is not exerting a major, direct effect on osteoblast proliferation and differentiation. Rather it is having an indirect secondary action to increased expression of JunB and c-Jun proteins, which will form heterodimers with freely available ectopic c-Fos proteins to modulate the effects of ectopic c-Fos.

The mechanisms by which exogenous c-Fos may inhibit BMP-2-induced alkaline phosphatase activity is not clear. The alkaline phosphatase promoter contains AP-1 sites (Owen *et al*, 1990) and it is possible that when occupied they exert an inhibitory effect on the transcription of the alkaline phosphatase gene. Alternatively, c-Fos may modulate BMP-2 effects by affecting the expression of BMP receptors, or the inhibitory SMADs (e.g., SMAD-6, SMAD-7). Interactions with other transcription factors, most notably Cbfa1/runx2, may also contribute to the observed effects. BMP-2 can induce Cbfa1 expression in various osteoblast cell lines to coordinate osteoblast differentiation (Gori *et al*, 1999; Lee *et al*, 2000; reviewed in Yamaguchi *et al*, 2000), and c-Fos/c-Jun heterodimers and Cbfa1 can physically interact and regulate osteoblast gene expression (Winchester *et al*, 2000; Hess *et al*, 2001; Chinenov and Kerppola, 2001).

Clearly, further experiments need to be performed to address the potential mechanisms by which c-Fos may alter BMP-2 signalling in KT1.5 cells.

4.6 Conclusions

Inducible overexpression of c-Fos in MC3T3-E1 osteoblastic cells may result in an inhibition of the osteoblast phenotype *in vitro.* This was evidence by a decrease in BMP-2-induced alkaline phosphatase expression following ectopic c-Fos expression. Although these results are based on preliminary data, the mechanisms of this may potentially be mediated through a small increase in proliferation and inhibition of differentiation of cells.

5. A role for c-Fos on osteoblast apoptosis

5.1 Introduction

Previous gain-of-function studies using H2-c-*fos*LTR transgenic mice found that apoptosis in c-Fos expressing transformed osteoblasts was increased relative to wild-type osteoblasts, but was maintained at relatively low levels throughout later stages of tumour formation, when high Bcl-2 levels were evident (Grigoriadis *et al*, 1993; El-Emir PhD thesis). In addition, osteoblast like cell lines derived from late stage bone tumours of H2-c-*fos*LTR mice did not readily undergo apoptosis following serum withdrawal or treatment with TNF-α. From this work it was postulated that during the earlier stages of tumour formation, ectopic c-Fos expression in transgenic mice leads to increased apoptosis in osteoblasts, but that there was an overall disruption of the balance between proliferation and apoptosis in the osteoblasts leading to malignant transformation.

To gain further insights into the role of ectopic c-Fos expression in osteoblast apoptosis prior to osteoblast transformation, KT1.5 cells (described in Chapter 4) were used. These cells were used to study the effects of c-Fos overexpression on apoptosis in osteoblastic cells because they are a non-transformed cell line and considered to represent osteoblastic cells at an early stage of osteoblast differentiation.

5.2 Effects of exogenous c-Fos expression on osteoblast apoptosis

Cells were grown under standard conditions in the presence or absence of Tc to induce expression of exogenous c-Fos. Apoptosis was induced by serum withdrawal or serum withdrawal in the presence of TNF-α, and compared to control growth medium containing 10% serum. The response of KT1.5 cells to different concentrations of TNF-α under reduced serum conditions was initially determined for 24 or 48 hours treatment to induce apoptosis. From these results, 48 hours treatment at 30ng/ml produced 50% apoptosis and was considered a suitable concentration for further experiments (data not shown).

Cell morphology following treatment was first monitored by light microscopy. Cells grown in the absence of serum began to show a change in morphology after 48 hours compared with those observed grown in medium containing 10% serum. Typically the cells retracted from the substrate, rounded up and eventually detached from the culture dish (Figure 5.1 A). The numbers of floating cells appeared to be

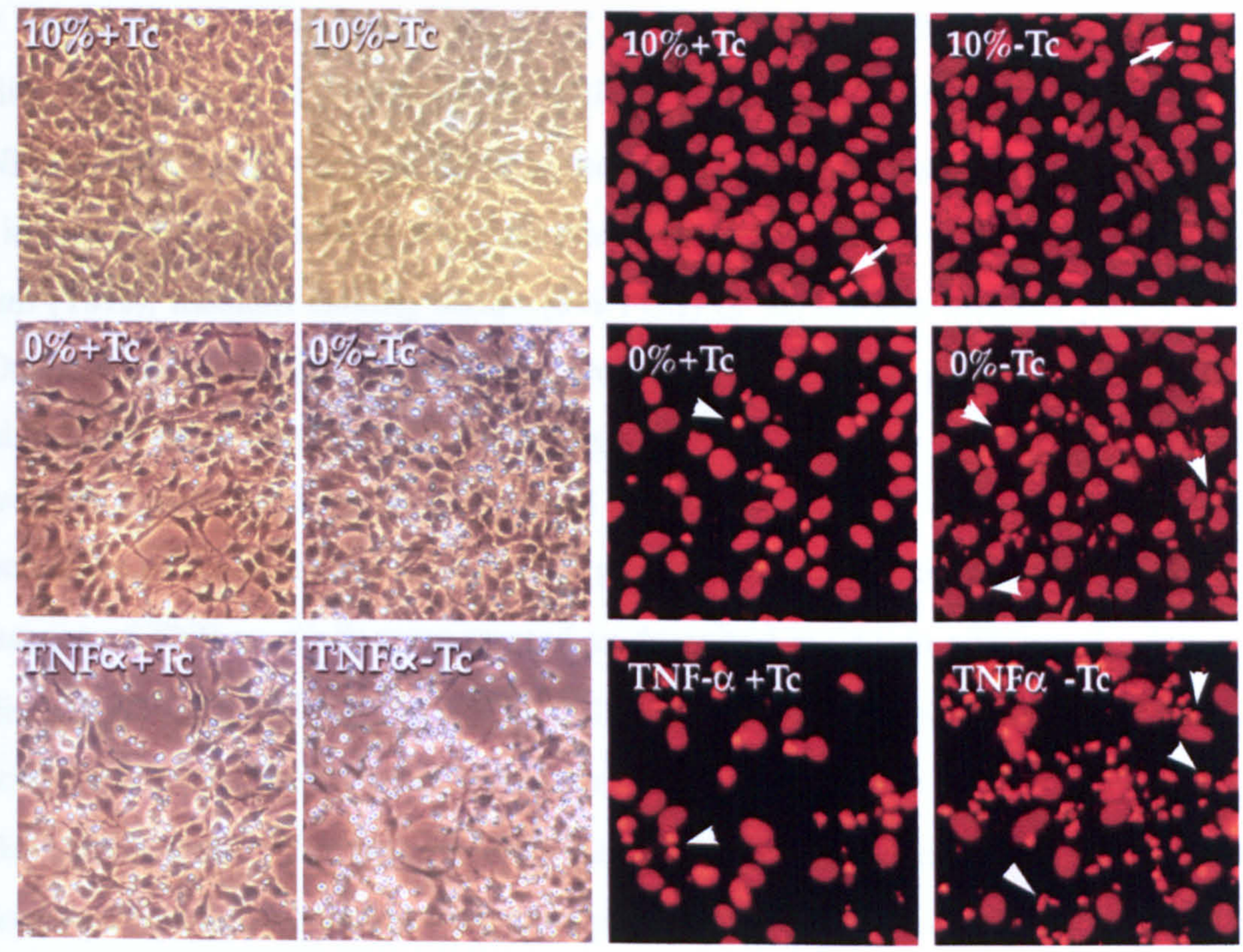
greater in the absence of Tc, i.e. when c-Fos was expressed, than that in the presence of Tc. This appeared to be further potentiated in cells treated with TNF-α.

To characterise further this apoptotic effect, cells were fixed and stained with propidium iodide (PI) to quantify apoptotic nuclei under fluorescence microscopy. Morphological changes which are a hallmark of apoptosis such as cell shrinking, nuclear condensation, and nuclear fragmentation can be immediately scored using fluorescence microscopy and the percentage of apoptotic cells calculated. Figure 5.1 shows the pro-apoptotic effects of ectopic c-Fos protein in KT1.5 cells following serum deprivation and TNF-α treatment. Very few, if any, apoptotic nuclei were present in cells cultured in 10% serum and there were no significant differences in apoptotic nuclei between Tc treatments (Figure 5.1 B). Serum withdrawal clearly induced apoptosis, and could be observed when cells were grown in the absence as well as in the presence of Tc. However, when ectopic c-Fos was expressed (i.e. in the absence of Tc), there was a significant increase (~1.5-fold) in the number of apoptotic cells when compared with cells grown in the presence of Tc (data represented in Figure 5.1 B).

When cells were induced to undergo apoptosis in the presence of TNF-α and absence of serum, there was a marked increase in apoptosis in both the presence and absence of Tc, that was of similar magnitude in both treatment groups (Figure 5.1 B). Moreover, when KT1.5 cells were cultured under normal serum conditions and treated with TNF-α, the cells were less sensitive to TNF-α than under reduced serum conditions (Figure 5.1 B).

As controls, KT2.1 cells (a luciferase expressing clone; see Chapter 4) were also induced to undergo apoptosis by serum withdrawal and TNF-α treatment. Levels of apoptosis in the presence and absence of Tc were similar in both the presence and absence of TNF-α, indicating that the withdrawal of Tc alone had no effect on apoptosis (Figure 5.2).

A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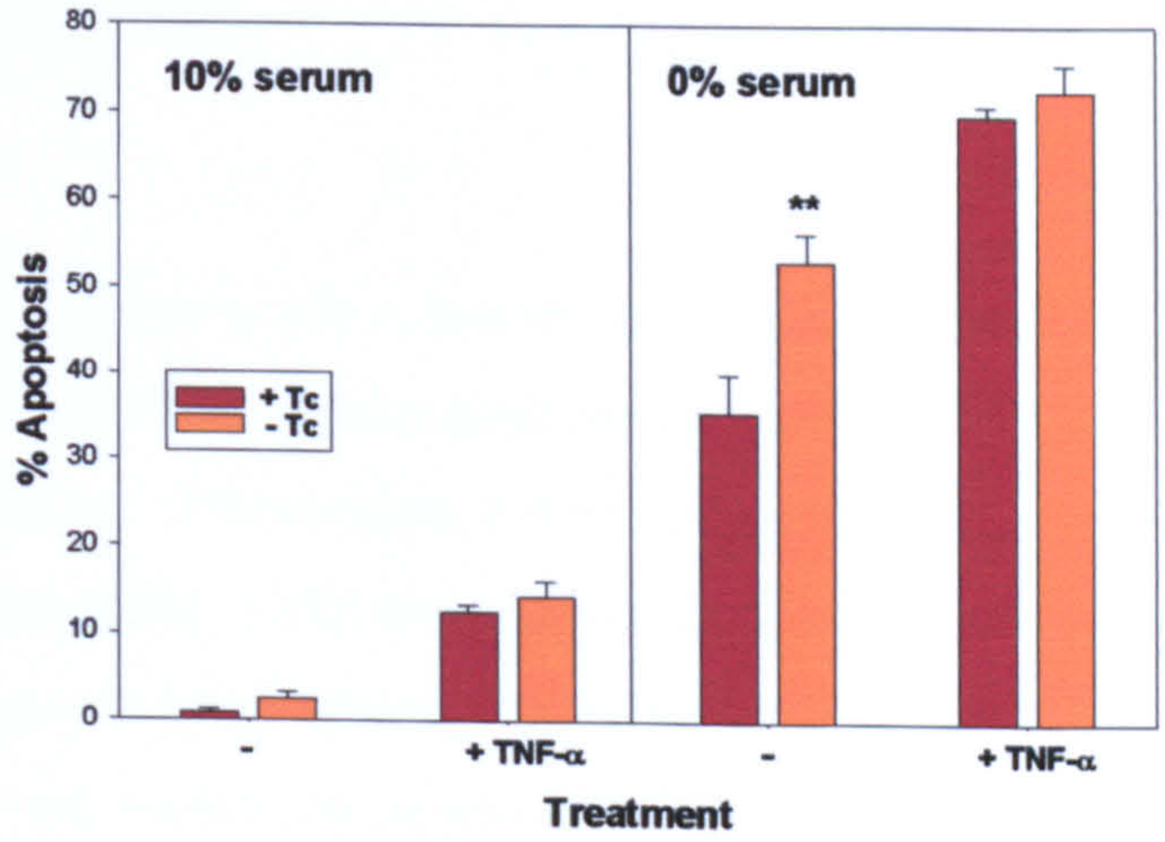
B


10% serum
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% Apoptosis
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- Tc
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30
20
10
0
-
+ TNF-α
-
+ TNF-α
Treatment

Figure - 5.1 – Ectopic c-Fos expression sensitises cells to apoptosis induced by serum withdrawal and TNF-α. (A) Morphological changes and propidium iodide staining of KT1.5 cells grown in the absence of serum and in the presence of TNF-α. KT1.5 cells were cultured in the presence and absence of Tc for 48 hours to induce c-Fos expression. Medium was then replaced with complete growth medium (10%), medium lacking serum (0% FCS) or serum-free medium plus TNF-α (30ng/ml). After 48 hours, cells were fixed and stained with propidium iodide (PI) and viewed using fluorescence microscopy. Arrows indicate -mitotic cells and arrowheads denote apoptotic nuclei. Total magnification: x200. (B) Propidium iodide assessment of apoptosis of KT1.5 cells grown in the absence of serum and in the presence of TNF-α. Cells were treated as described in (A); in addition the data for cells treated with TNF-α (30ng/ml) in 10% serum have been shown. Apoptotic nuclei were quantified by counting at least 100 PI-stained cells per field in 5 random fields per coverslip. The mean % apoptosis (± S.D.) of triplicate samples are shown. These results are a representative example of at least three separate experiments. (**, $p<0.01$, compared to corresponding +Tc).

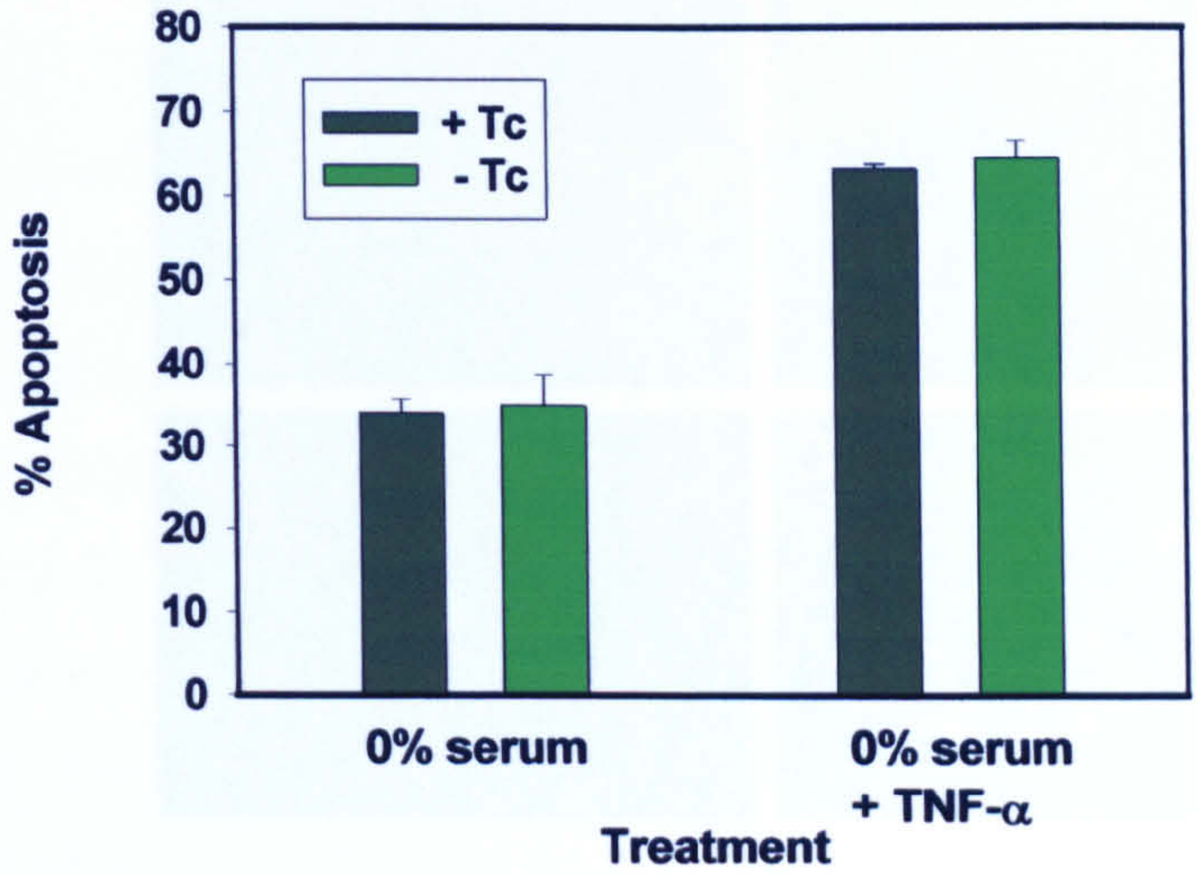


Figure 5.2 – Stimulation of apoptosis under reduced serum conditions and TNF-α in KT2.1 cells. Propidium iodide assessment of apoptosis of KT2.1 cells grown in the absence of serum and in the presence of TNF-α. KT2.1 cells were treated as described in Figure 5.1. The means (± S.D.) of triplicate samples are shown. Cells treated with complete growth medium (10%) showed 0% apoptosis and have not been shown here. These results are a representative example of two independent experiments.

In order to further confirm that the cell death observed was apoptotic in nature, DNA strand breaks were determined by terminal deoxynucleotidyl transferase end labelling (TUNEL). This method detects DNA fragmentation and is indicative of a late stage of apoptosis. Cells were grown on glass coverslips under standard conditions in the presence and absence of Tc to induce expression of exogenous c-Fos before serum was withdrawn as above. Expression of exogenous c-Fos clearly increased the numbers of TUNEL positive cells following serum withdrawal as fragmented and condensed nuclei were clearly distinguishable using this method (Figure 5.3). To confirm the specificity of the assay the TUNEL reaction was carried out in the absence of TdT-transferase. Under these conditions, cells exhibiting fluorescence could not observed.

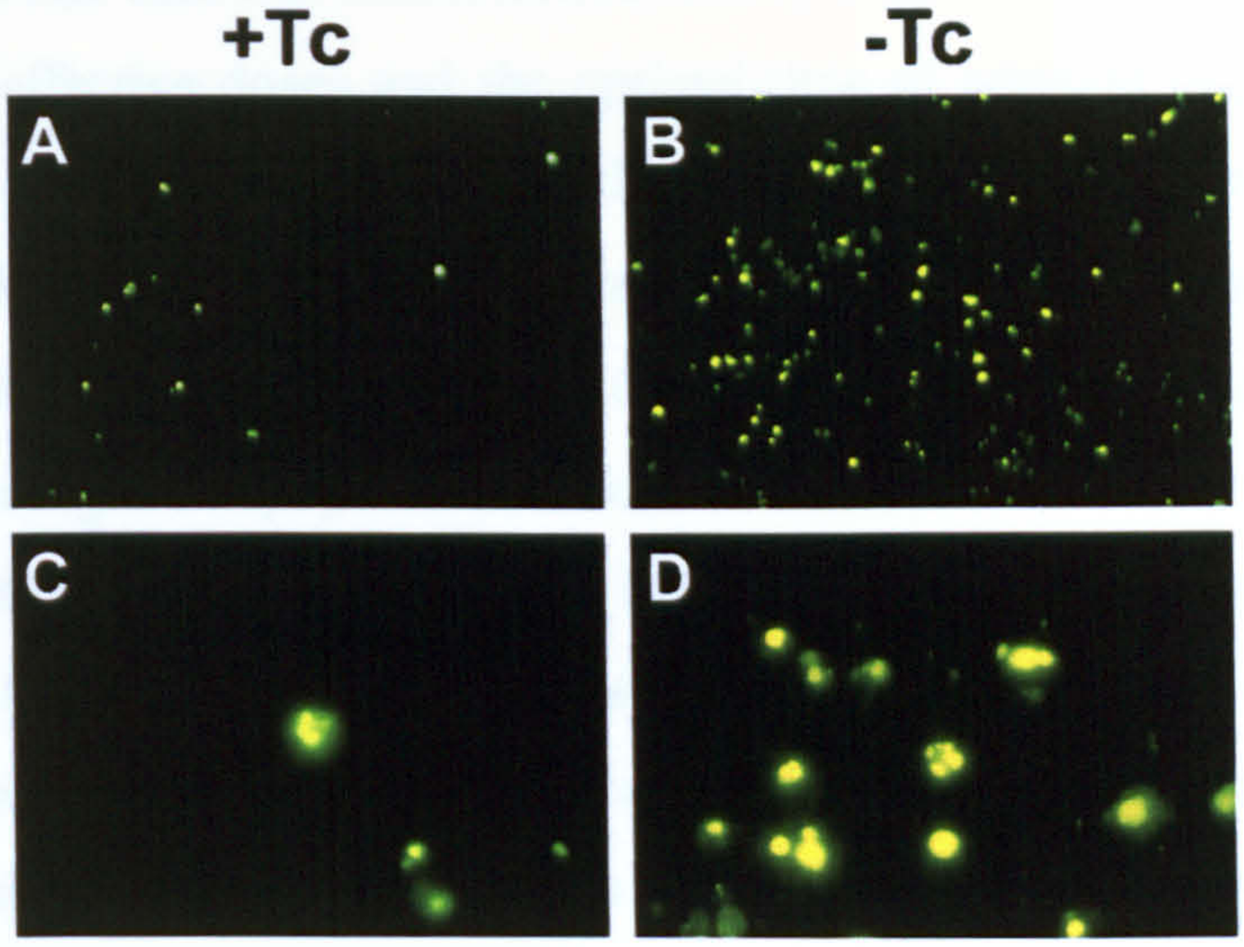


Figure 5.3 - Assessment of c-Fos-induced apoptosis of KT1.5 cells via TUNEL staining. KT1.5 cells were cultured on glass coverslips as in Fig. 5.1 in the presence (A, C) and absence (B, D) of Tc for 48 hours to induce ectopic c-*fos* expression, before serum was withdrawn. Cells were fixed and stained using the TUNEL technique as described in section 2.6.11. Only cells grown in 0% serum have been depicted due to absence of apoptotic nuclei in normal growth medium. Total magnification: x200 (A, B) and x600 (C, D).

Taken together, these results show that serum deprivation induces apoptosis in KT1.5 cells which is potentiated by expression of exogenous c-Fos. TNF-α can enhance this apoptosis but it is not potentiated further by ectopic c-Fos expression.

5.2.1 Effect of Caspase inhibitors on c-Fos-induced apoptosis

To investigate the mechanism of c-Fos induced apoptosis, inhibitors of steps in the apoptosis pathway were used. Caspases play a well established role in the execution phase of apoptosis (see section 1.5.1), although caspase-independent apoptotic pathways also exist (e.g., Gross *et al*, 1999). The irreversible, broad-spectrum caspase-I-like inhibitor, Z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-O-methyl-fluoromethyl ketone), was used to try to inhibit c-Fos induced apoptosis. This chemical has been shown to be an effective inhibitor of chemical and receptor-mediated apoptosis via inhibition of caspases 1, 3, 4, and 7 in a wide range of cell types (e.g., Cohen, 1997; Robertson *et al*, 2000; Johnson *et al*, 1999), including osteoblasts (Pucci *et al*, 1999).

The effect of Z-VAD-fmk was characterised first by a series of experiments aimed at establishing the effective doses and the optimal time of addition (Figure 5.4). To this end, KT1.5 cells were initially cultured in the presence of Tc in standard conditions, before cells were cultured both in the presence or absence of serum, and in a range of Z-VAD-fmk concentrations (0, 10, 30, 100μM). After 24 hours, cultures were assessed for signs of apoptosis as described above.

Z-VAD-fmk did not inhibit apoptosis induced by serum withdrawal (Figure 5.4 B). The reason for this may have been that even though Z-VAD-fmk is a highly cell permeable compound, cells need to be pretreated with Z-VAD-fmk before apoptosis is induced. Thus, subsequent experiments were set up which incorporated various regimes of pretreatment, as described in Figure 5.4 A.

To determine whether the pro-apoptotic effect of c-Fos was mediated through caspase activation, cells were pretreated with Z-VAD-fmk prior to stimulation of apoptosis. To confirm that Z-VAD-fmk was active in inhibiting apoptosis, the inhibition of TNF-α induced apoptosis was used a positive control. Z-VAD-fmk has been shown to alleviate TNF-α induced apoptosis in human prostate cancer cells (e.g., Kimura and Gelman, 2000). Cells were cultured in the presence and absence of Tc to induce c-Fos expression, and then treated for 24 hours with Z-VAD-fmk (10μM). They were then treated for a further 48 hours with Z-VAD-fmk (10μM) and complete growth medium, serum withdrawal, or serum withdrawal plus TNF-α (Figure 5.4 C). Interestingly, assessment of apoptosis demonstrated that using these conditions, Z-VAD-fmk did not inhibit apoptosis induced either by c-Fos, serum withdrawal, or TNF-α.

To further investigate whether Z-VAD-fmk could inhibit apoptosis, the pretreatment period was reduced to 1 or 6 hours. In the presence of ectopic c-Fos expression, pretreatment of cells with Z-VAD-fmk for either 1 or 6 hours showed no protection from apoptosis induced by serum withdrawal (Figure 5.4 D). In the presence of Tc, there was a small and significant reduction in induction of apoptosis observed in cells treated with Z-VAD-fmk. Overall, therefore, Z-VAD-fmk showed no protection from apoptosis induced by ectopic c-Fos expression in KT1.5 cells.

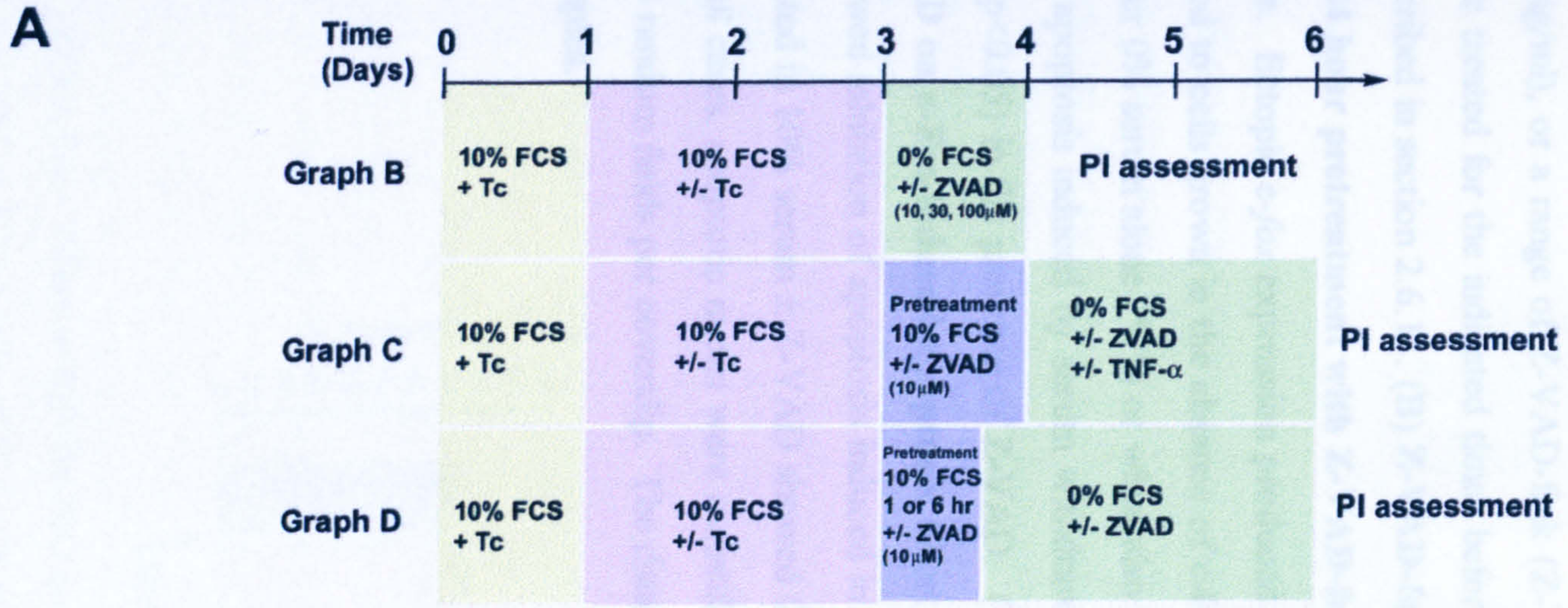
To further investigate potential caspase pathways involved in c-Fos induced apoptosis, another caspase inhibitor, acetyl DEVD aldehyde (DEVD-CHO) was used, which inhibits caspases-3, 6, 7 and 10. DEVD-CHO has been shown to inhibit

chemical and growth factor induced apoptosis in osteoblasts (Plotkin *et al*, 1999; Kawakami *et al*, 1998) and a variety of other cell types (e.g., Cohen, 1997; Schlegel *et al*, 1996).

Cells were cultured as for previous apoptosis experiments and pretreated for 1 hour with DEVD-CHO, before apoptosis was induced by serum withdrawal. The apoptosis induced by ectopic c-Fos was slightly but significantly reduced from 1.5-fold stimulation to 1.3-fold stimulation in the presence of 30μM DEVD-CHO, with no effects on basal apoptosis levels (Figure 5.5). This inhibitory effect of DEVD-CHO also appeared to be dose-dependent, but further work using a wider concentration range of DEVD-CHO will be required.

Cells were also pretreated with DEVD-CHO (30μM) as above before induction of apoptosis by serum withdrawal in the presence of TNF-α. DEVD-CHO significantly reduced the apoptosis induced by TNF-α in the absence of serum by 37% in cells cultured in the absence of c-Fos (Figure 5.6). However, no inhibition of apoptosis was observed in the presence of c-Fos. This suggests that only the apoptosis induced by TNF-α can be inhibited under these conditions and the presence of ectopic c-Fos is forcing the cells into a condition which cannot be overridden by caspase inhibitors.

Overall, these results show that while a slight inhibition in c-Fos-induced apoptosis was observed using DEVD-CHO, the overall effects using both caspase inhibitors, Z-VAD-fmk and DEVD-CHO, showed no marked inhibition, suggesting that the c-Fos effects on apoptosis may be caspase-independent.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Time (Days)
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1
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Graph B
10% FCS + Tc
10% FCS +/- Tc
0% FCS +/- ZVAD (10, 30, 100μM)
PI assessment
Graph C
10% FCS + Tc
10% FCS +/- Tc
Pretreatment 10% FCS +/- ZVAD (10μM)
0% FCS +/- ZVAD +/- TNF-α
PI assessment
Graph D
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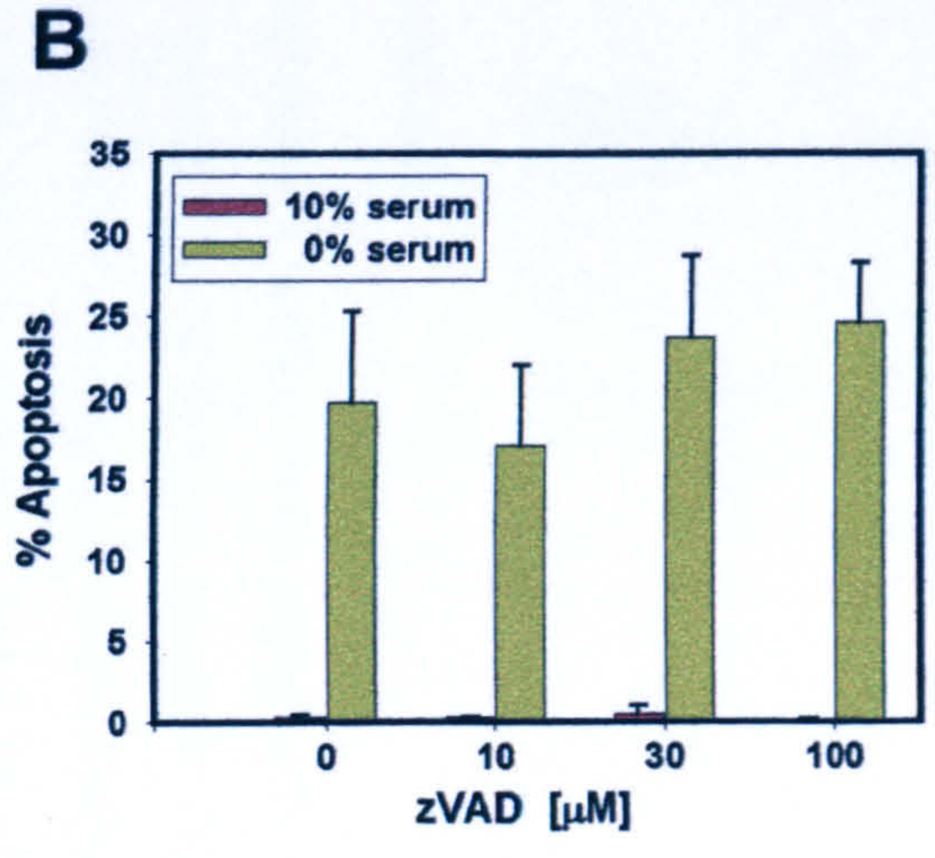
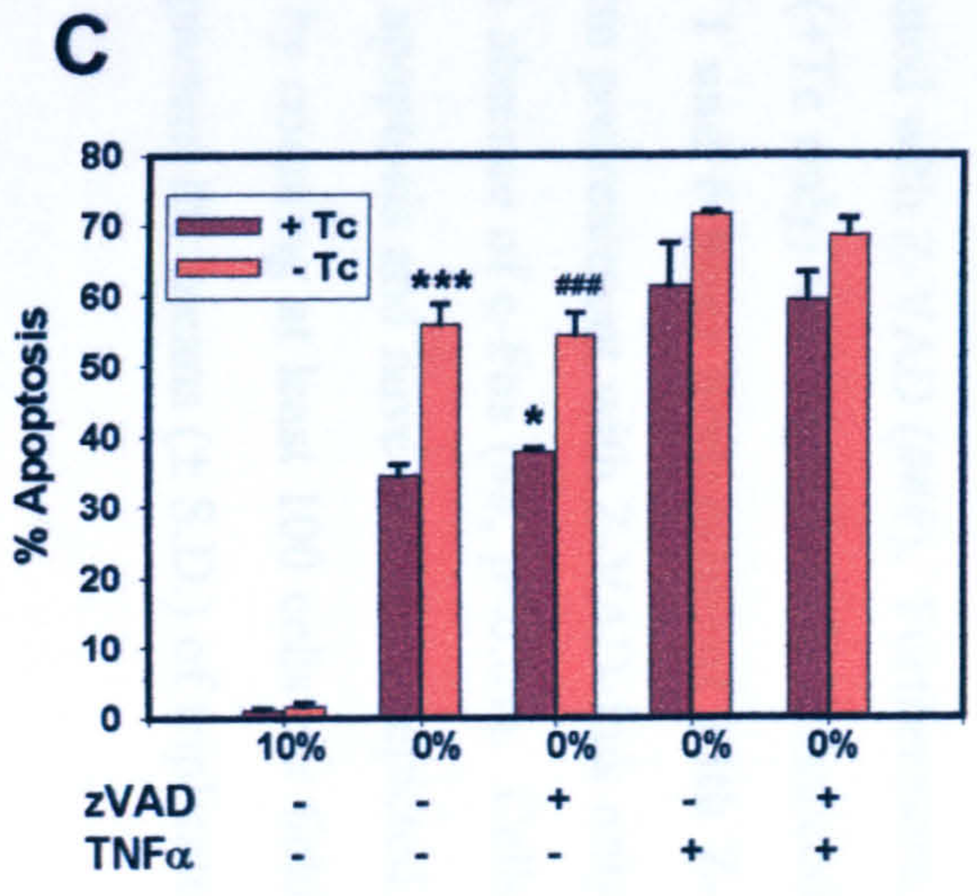
10% FCS +/- Tc
Pretreatment 10% FCS 1 or 6 hr +/- ZVAD (10μM)
0% FCS +/- ZVAD
PI assessment


B
10% serum
0% serum
% Apoptosis
zVAD [μM]
0
10
30
100


C
+ Tc
- Tc
% Apoptosis
***
###
*
10%
0%
0%
0%
0%
zVAD
TNFα

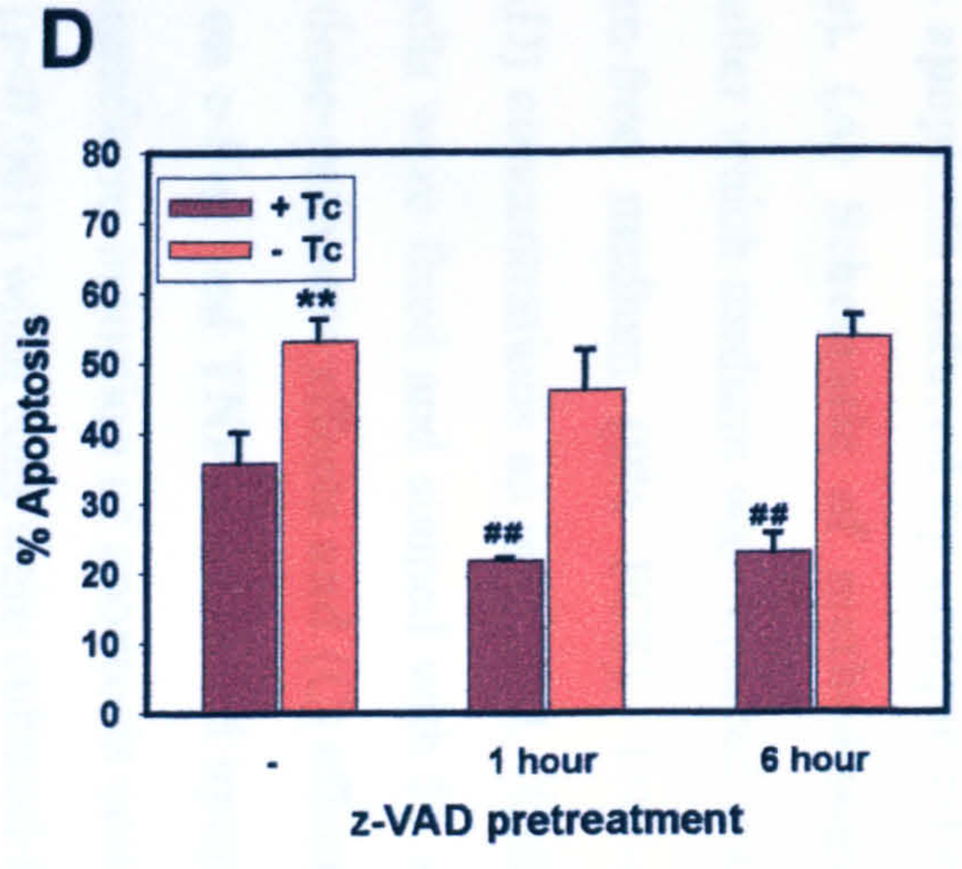

D
+ Tc
- Tc
% Apoptosis
**
##
##
-
1 hour
6 hour
z-VAD pretreatment

Figure 5.4 – Effects of Z-VAD-fmk on apoptosis induced by ectopic c-Fos expression in KT1.5 cells (previous page). (A) Schematic of methodology. KT1.5 cells were cultured ±Tc for 48 hours, after which medium was replaced with complete growth medium (10%) or serum-free medium (0% FCS), TNF-α (30ng/ml), or a range of Z-VAD-fmk (Z-VAD) concentrations as indicated. Cells were treated for the indicated times before cells were fixed and stained with PI as described in section 2.6.11. (B) **Z-VAD-fmk dose-response effects and** (C) **effects of 24 hour pretreatment with Z-VAD-fmk on c-Fos and TNF-α-induced apoptosis.** Ectopic c-*fos* expression produced a significant induction of apoptosis compared to cells grown in the absence of c-Fos ($p<0.001$) when cells were cultured in either 0% serum alone (***) or when also treated with Z-VAD (###). Furthermore, the apoptosis induced by serum withdrawal (+Tc only) was significantly enhanced (*, $p<0.05$) in the presence of Z-VAD. (D) **1 and 6 hour pretreatment with Z-VAD on c-Fos-induced apoptosis.** Short term pretreatment with Z-VAD-fmk only showed inhibition of apoptosis induced in the absence of c-Fos (##, $p<0.01$). Cells treated in 10% serum ± Z-VAD showed 0% apoptosis and have not been depicted. In all cases, apoptotic nuclei were quantified by counting at least 100 cells per field in 5 random fields per coverslip. The data represent the means (± S.D.) of triplicate samples.

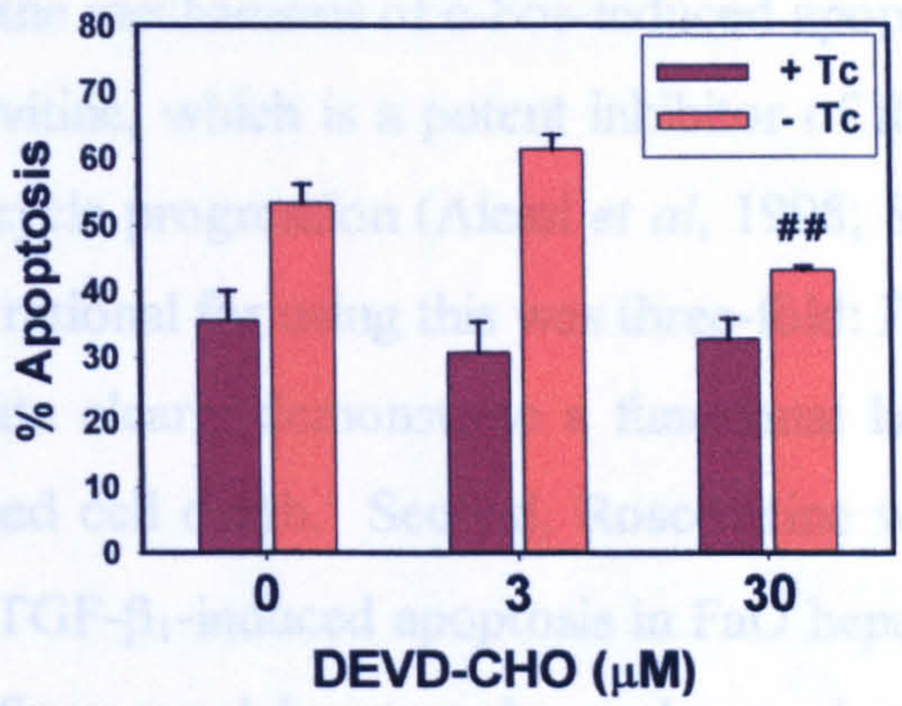


Figure 5.5 – Effect of the caspase-3 inhibitor DEVD-CHO on apoptosis induced by serum withdrawal and ectopic c-Fos in KT1.5 cells. Cells were cultured in the presence and absence of Tc for 48 hours to induce c-Fos expression. Cells were pretreated for 1 hour with the DEVD-CHO as indicated, before medium was replaced with complete growth medium (10%), serum-free medium (0% FCS) plus DEVD-CHO, ± Tc. After 48 hours cells were stained with PI and apoptotic cells were quantified as in Figure 5.4. The means (± S.D.) of triplicate samples are shown. Cells treated with 10% serum ± DEVD-CHO showed 0% apoptosis and have not been depicted. (## p=0.01, compared to corresponding –Tc treatment at 0 and 3μM DEVD-CHO).

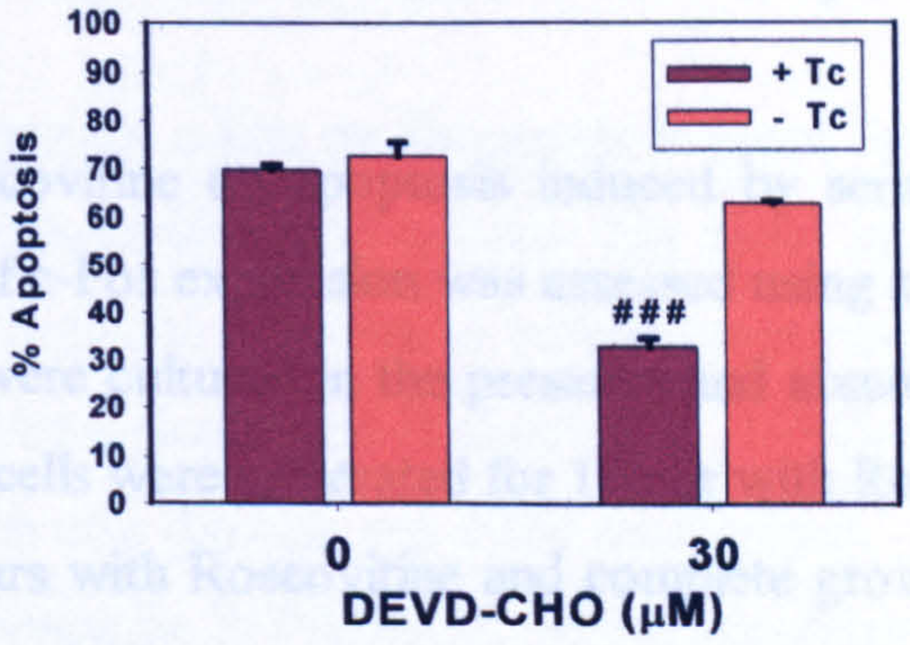


Figure 5.6 – Effect of DEVD-CHO on apoptosis induced by serum withdrawal, c-Fos and TNF-α KT1.5 cells. KT1.5 cells were cultured ±Tc for 48 hours to induce c-Fos expression. Cells were pretreated for 1 hour with DEVD-CHO as indicated, before medium was replaced with serum-free medium (0% FCS) plus TNF-α (30ng/ml), and ± DEVD-CHO as indicated. Cells were treated for further 48 hours before PI assessment and quantification of apoptosis as above. The means (± S.D.) of triplicate samples are shown which are representative of one experiment. (### p=0.001, compared to corresponding +Tc).

5.2.2 Cell cycle inhibition by Roscovitine prevents c-Fos induced apoptosis

To further understand the mechanisms of c-Fos-induced apoptosis, KT1.5 cells were pretreated with Roscovitine, which is a potent inhibitor of the cyclin-dependent kinase, CDK2, and cell cycle progression (Alessi *et al*, 1998; Schutte *et al*, 1997; see also section 1.4). The rational for using this was three-fold: First, as described in the introduction, recent data clearly demonstrate a functional link between cell proliferation and programmed cell death. Second, Roscovitine was chosen since it has been shown to inhibit TGF-β_1-induced apoptosis in FaO hepatoma cells (Choi *et al*, 1999). Finally, data from our laboratory have shown that under reduced serum conditions, c-Fos can increase CDK2 activity and cause accelerated cell cycle progression (Sunters *et al*, 2000). Dose-response experiments were performed initially to establish the optimal concentration of Roscovitine for inhibition of cell cycle progression. KT1.5 cells were cultured in standard medium, in the presence and absence of Tc and Roscovitine for 48 hours and the cell density roughly estimated by methylene blue staining. Following a qualitative assessment after 24 hours, there appeared to be no difference in cell number, however, after 48 hours fewer cells could be observed in the cultures treated with 30μM Roscovitine (pictures not shown). No obvious differences in cell density were apparent between ± Tc cultures. Thus, the cell growth in KT1.5 cells was reduced by Roscovitine at approximately 30μM.

The effect of Roscovitine on apoptosis induced by serum withdrawal in the presence and absence of c-Fos expression was assessed using a range of Roscovitine concentrations. Cells were cultured in the presence and absence of Tc to induce c-Fos expression, before cells were pretreated for 1 hour with Roscovitine. Cells were then treated for 48 hours with Roscovitine and complete growth medium or serum withdrawal (Figure 5.7). Cells treated with Roscovitine in standard growth medium showed no induction of apoptosis (data not shown). Following serum withdrawal, c-Fos caused a 2.8-fold stimulation in the percentage of apoptotic cells (Figure 5.7). Treatment with Roscovitine significantly attenuated the apoptosis induce by serum deprivation in the presence of ectopic c-Fos expression, at all concentrations tested. This suggests that Roscovitine can inhibit c-Fos-induced apoptosis, possibly in a cell cycle-dependent way.

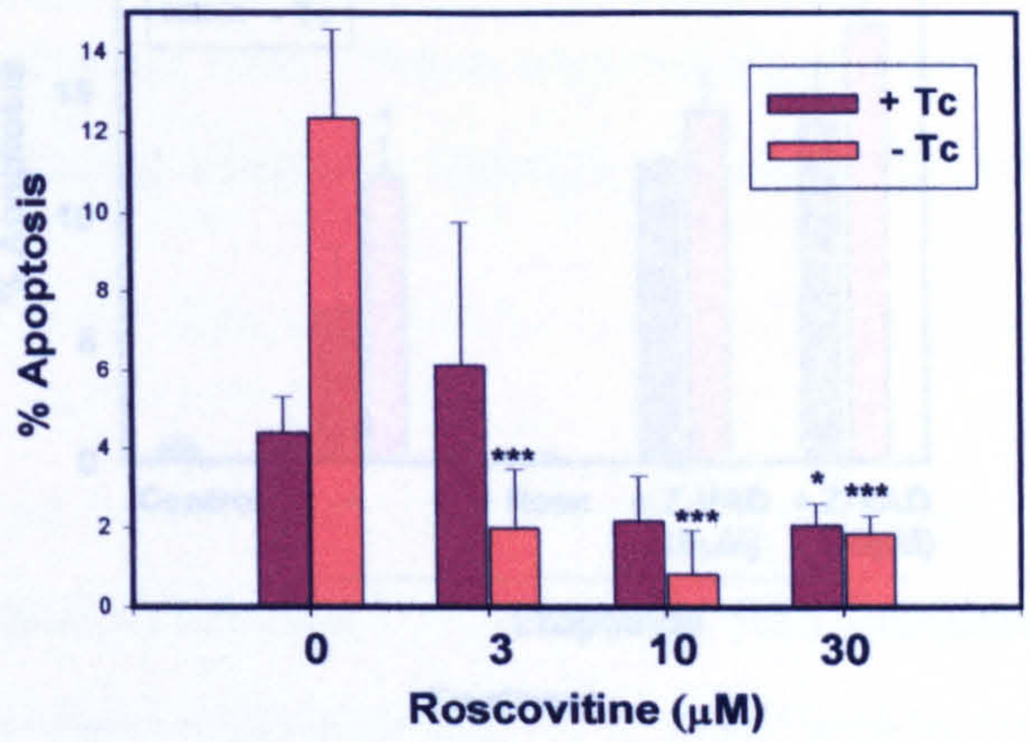


Figure 5.7 – Effects of Roscovitine on apoptosis induced by serum withdrawal and c-Fos in KT1.5 cells. Cells were cultured ± Tc for 48 hours to induce c-Fos expression. Cells were pretreated for 1 hour with Roscovitine as indicated, before apoptosis was induced by serum withdrawal (0% FCS) in the presence and absence of Roscovitine. Cells were treated for further 48 hours before PI assessment as described above. The means (± S.D.) of triplicate samples are shown. Results for 30μM are representative of at least three separate experiments. *** $p \leq 0.001$ and * $p < 0.05$ compared to respective ±Tc 0μM Roscovitine control.

The effect of exogenous c-Fos on Etoposide-induced apoptosis under normal serum conditions was investigated, and whether this could be inhibited by Roscovitine or Z-VAD-fmk. Etoposide is known to induce apoptosis by forming a complex between DNA and DNA topoisomerase II (Lock *et al*, 1994), and is thus an apoptosis inducing agent which acts through cell cycle mechanisms. Cells were pretreated for 1 hour with Roscovitine or Z-VAD-fmk, before apoptosis was induced by Etoposide in the presence and absence of Roscovitine or Z-VAD-fmk, all in the presence and absence of Tc. Etoposide-induced apoptosis in KT1.5 cells was significantly stimulated 2-fold by the presence of exogenous c-Fos (Figure 5.8). Roscovitine abolished the apoptosis induced by both Etoposide alone and Etoposide in the presence of c-Fos to less than 1% apoptosis for both groups. However, treatment with Z-VAD-fmk showed no effects on inhibition of apoptosis either in the presence or absence of c-Fos (Figure 5.8).

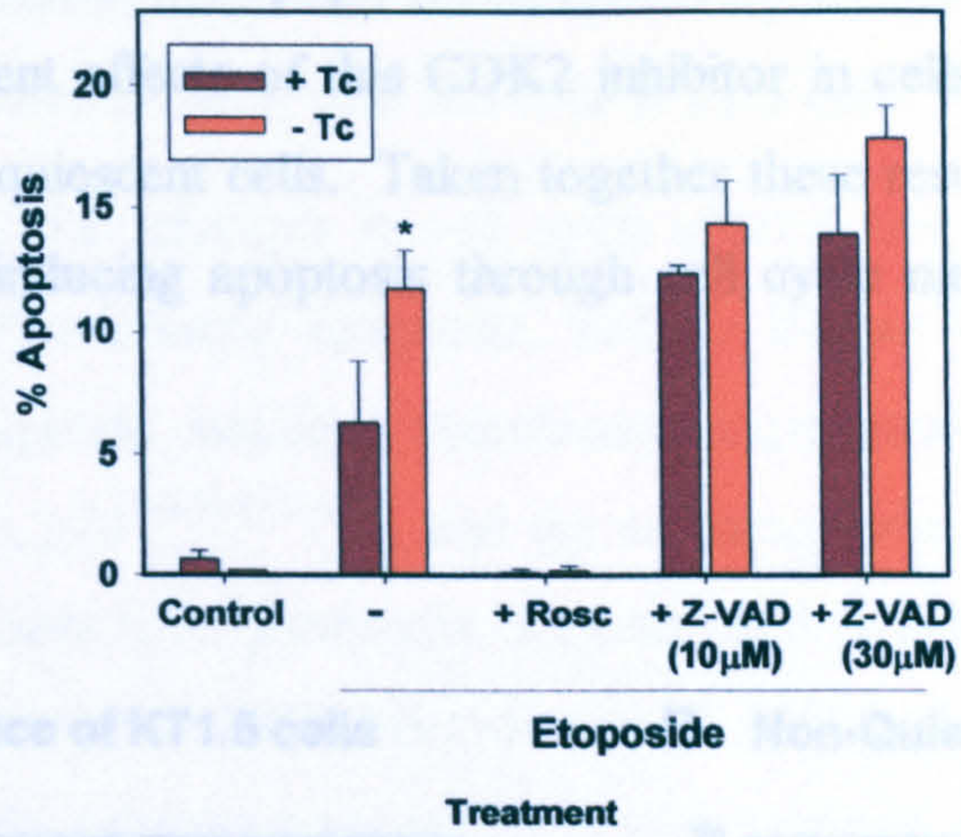


Figure 5.8 – Roscovitine but not Z-VAD-fmk inhibits apoptosis induced by Etoposide. KT1.5 cells were cultured in the presence and absence of Tc for 48 hours to induce c-Fos expression. Cells were pretreated for 1 hour with DMSO vehicle control, Roscovitine (30μM) or Z-VAD-fmk (10, 30μM) as indicated, before apoptosis was induced by Etoposide (100μg/ml) in the presence and absence of Roscovitine or Z-VAD-fmk. Cells were treated for a further 24 hours before cells were fixed and stained with PI. Apoptotic nuclei were quantified by counting at least 100 cells per field in 5 random fields per coverslip. The means ± S.D. of triplicate samples are shown from a representative experiment of two separate experiments. *$p<0.05$ compared to +Tc Etoposide control.

To confirm that Roscovitine was reducing the apoptosis induced by c-Fos by cell cycle dependent mechanisms, KT1.5 cells were made quiescent and synchronised by treatment in 0.5% serum before induction of apoptosis. In the presence of ectopic c-Fos there was a significant increase in the numbers of apoptotic nuclei in quiescent K1.5 cells (Figure 5.9 A, left panel). Interestingly, pretreatment with Roscovitine not only inhibited this increase, but completely reversed the induction of apoptosis by c-Fos (Figure 5.9 A, left panel). A similar pattern of results was found when cells were made quiescent and induced to undergo apoptosis by serum withdrawal, although the overall level of apoptosis was much greater under these conditions. This pattern of inhibition by Roscovitine was slightly different to that observed when cells were not made quiescent before induction of apoptosis. Figure 5.9 B shows the results from non-quiescent cells induced to undergo apoptosis as described previously for Figure 5.7. Under these conditions, Roscovitine inhibited

the c-Fos-induced increases in apoptosis to basal levels, but not further (Figure 5.9 B), indicating different effects of this CDK2 inhibitor in cells arrested in G_0 when compared with non-quiescent cells. Taken together these results support the possibility that c-Fos is inducing apoptosis through cell cycle mechanisms, specifically involving CDK2.

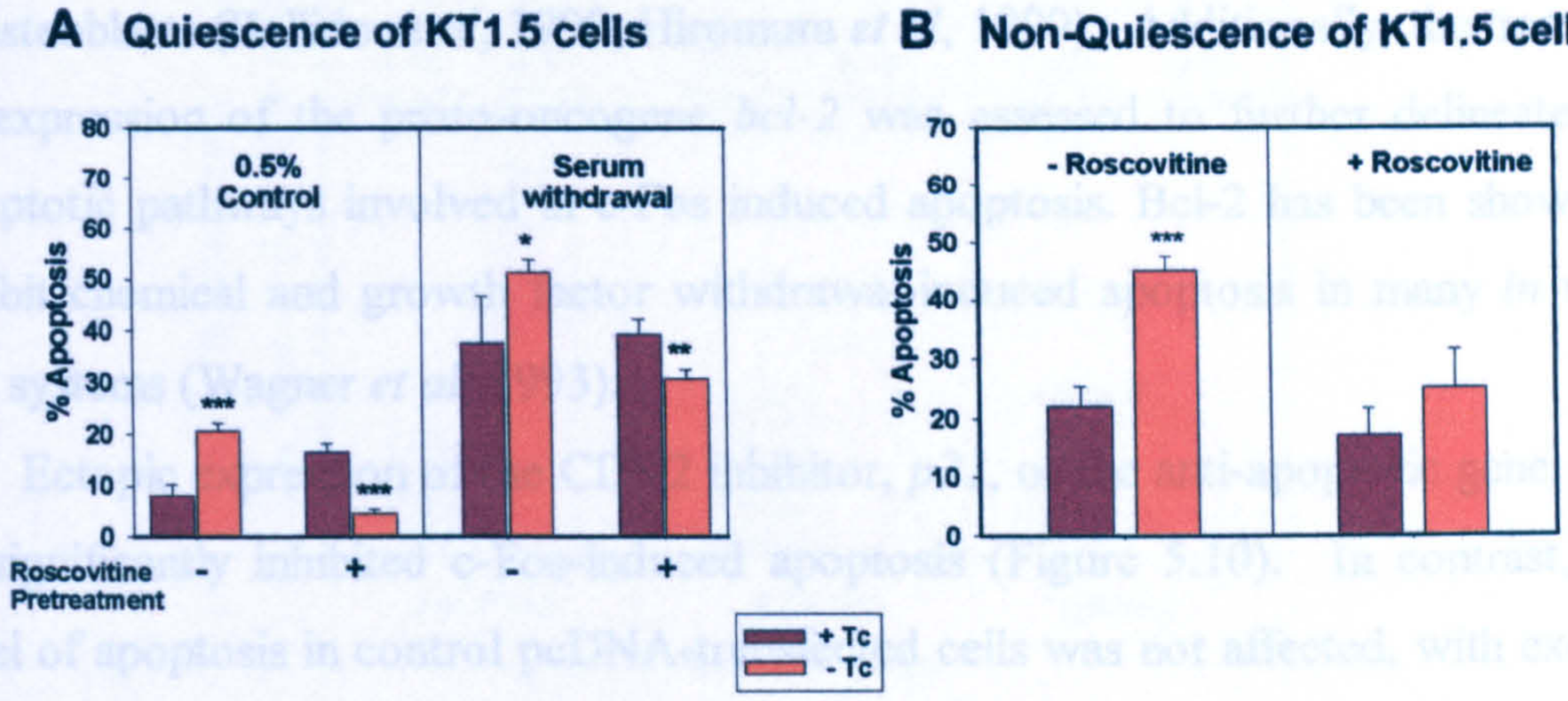


Figure 5.9 – Inhibition of apoptosis by Roscovitine in quiescent and synchronised KT1.5 cells compared to non-quiescent cells. (A) Cells were cultured on glass coverslips in the presence and absence of Tc for 24 hours to induce c-Fos expression before cells were pretreated for 24 hours 0.5% serum, then 24 hours ± Roscovitine (30μM) in 0.5% serum. Apoptosis was induced by serum withdrawal (0% FCS) or maintained in 0.5% serum, ± Roscovitine (30μM), ± Tc. Cells were treated for a further 48 hours before being fixed and stained with PI. (B) To control for previous observations with Roscovitine, cells were also treated as described for Figure 5.7, i.e. pretreatment ± Roscovitine (30μM), ± Tc in 10% serum for 24 hours, before induction of apoptosis by serum withdrawal ± Roscovitine, ± Tc. Apoptotic nuclei were quantified by counting at least 100 cells per field in 5 random fields per coverslip. The means ± S.D. of triplicate samples are shown. Data represents only one experiment. *$p \leq 0.05$, **$p < 0.01$, ***$p \leq 0.001$ compared to respective +Tc treatment.

5.2.3 c-Fos mediated apoptosis is abrogated by ectopic expression of the CDK2 inhibitor, p21, and Bcl-2 in KT1.5 cells

To further confirm that changes in cell cycle control and CDK2 activity may be contributing to c-Fos-induced apoptosis, and to further investigate the possible mechanisms of apoptosis, transient transfection experiments were performed with the CDK2 inhibitor, $p21^{WAF1,CIP1,SDI1}$, and the anti-apoptotic gene *bcl-2*. Expression of *p21* and $p27^{kip1}$ have been previously demonstrated to inhibit apoptosis, including in osteoblasts (Bellido *et al*, 1998; Hiromura *et al*, 1999). Additionally, the transient co-expression of the proto-oncogene *bcl-2* was assessed to further delineate the apoptotic pathways involved in c-Fos induced apoptosis. Bcl-2 has been shown to inhibit chemical and growth factor withdrawal-induced apoptosis in many *in vitro* cell systems (Wagner *et al*, 1993).

Ectopic expression of the CDK2 inhibitor, *p21*, or the anti-apoptotic gene, *bcl-2*, significantly inhibited c-Fos-induced apoptosis (Figure 5.10). In contrast, the level of apoptosis in control pcDNA-transfected cells was not affected, with exogenous c-Fos causing ~3-fold increase in the number of apoptotic cells (Figure 5.10). This data further suggest that c-Fos is inducing apoptosis through a cell cycle-dependent mechanism, perhaps by stimulating the cell cycle activity of cells in the absence of growth factors. This may also support the potential anti-proliferative effects of Bcl-2 during apoptosis that have been previously reported (Johnson *et al*, 1999), especially since the Bcl-2 levels remain unchanged in KT1.5 cells (see below).

5.2.4 The expression of Bcl-2 and Bax following c-Fos expression

The apoptotic response of a cell can vary depending on the relative levels of Bax and Bcl-2 present. The anti-apoptotic proto-oncogene *bcl-2* has been shown to inhibit apoptosis in a variety of systems *in vitro*, and is expressed in osteoblasts *in vivo* during normal development as well as during bone tumour formation, in particular in c-Fos transgenic mice (El-Emir *et al*, unpublished/PhD thesis; see section 5.1 above). To investigate whether the increased sensitivity of KT1.5 cells to apoptosis following c-Fos expression may be a result of changes in the expression of Bcl-2 or Bax proteins, Western blot analysis was performed in KT1.5 cells following c-Fos induction, and additionally in AT9.2 cells and control KT2.1 cells. The results

showed that Bcl-2 protein levels were generally low and remained unchanged irrespective of c-Fos induction, whereas Bax levels were generally higher (Figure 5.11). More importantly, the apparent ratio of Bax:Bcl-2 remained unchanged, possibly suggesting that Bcl-2 and Bax were not regulated directly by c-Fos in these cells.

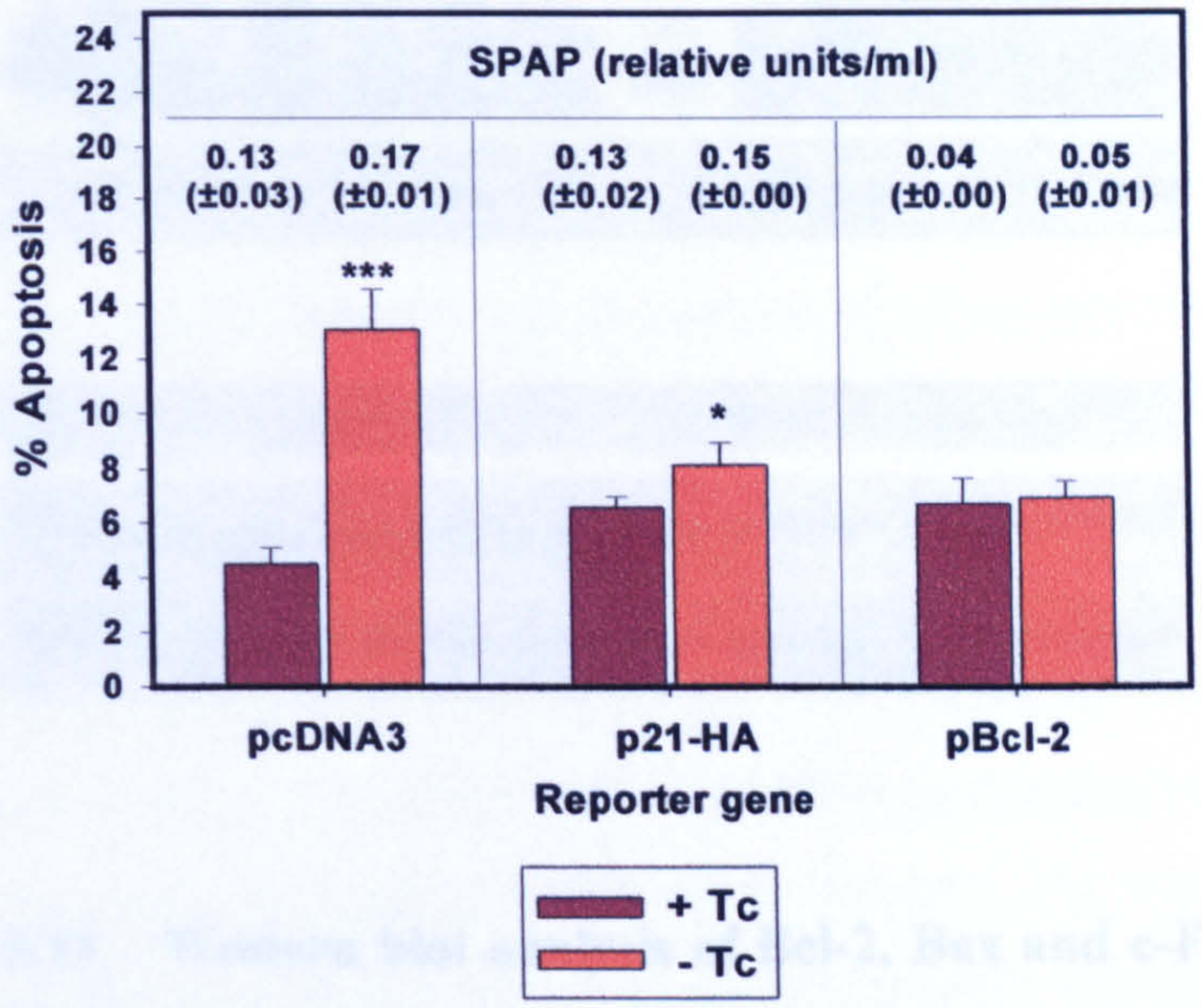


Figure 5.10 – Ectopic expression of p21 and Bcl-2 inhibits apoptosis induced by c-Fos following serum withdrawal in KT1.5 cells. Cells were transfected with pcDNA-3, p21-HA and pBcl-2 vectors or in the absence of DNA as described in section 2.6.6.1, and cells were cultured for further 24 hours in standard medium ± Tc. Apoptosis was induced by serum withdrawal (0% FCS), ± Tc for 24 hours before cells were fixed and stained with PI. Apoptotic nuclei were quantified by counting cells from 3 coverslips and at least 100 cells per field in 5 random fields. The expression vector pCMV-SPAP (section 2.6.6.3) was used as an internal control for transfection efficiency. SPAP activity, assayed 48 hours after transfection is shown, and is expressed as SPAP units/µl culture medium. Cells that had been treated with Effectene alone or no transfection treatment at all were also assessed for apoptosis; similar results were found as for cells transfected with pcDNA3 and have not been depicted. *** $p < 0.001$ and * $p < 0.05$ compared to respective +Tc treatment.

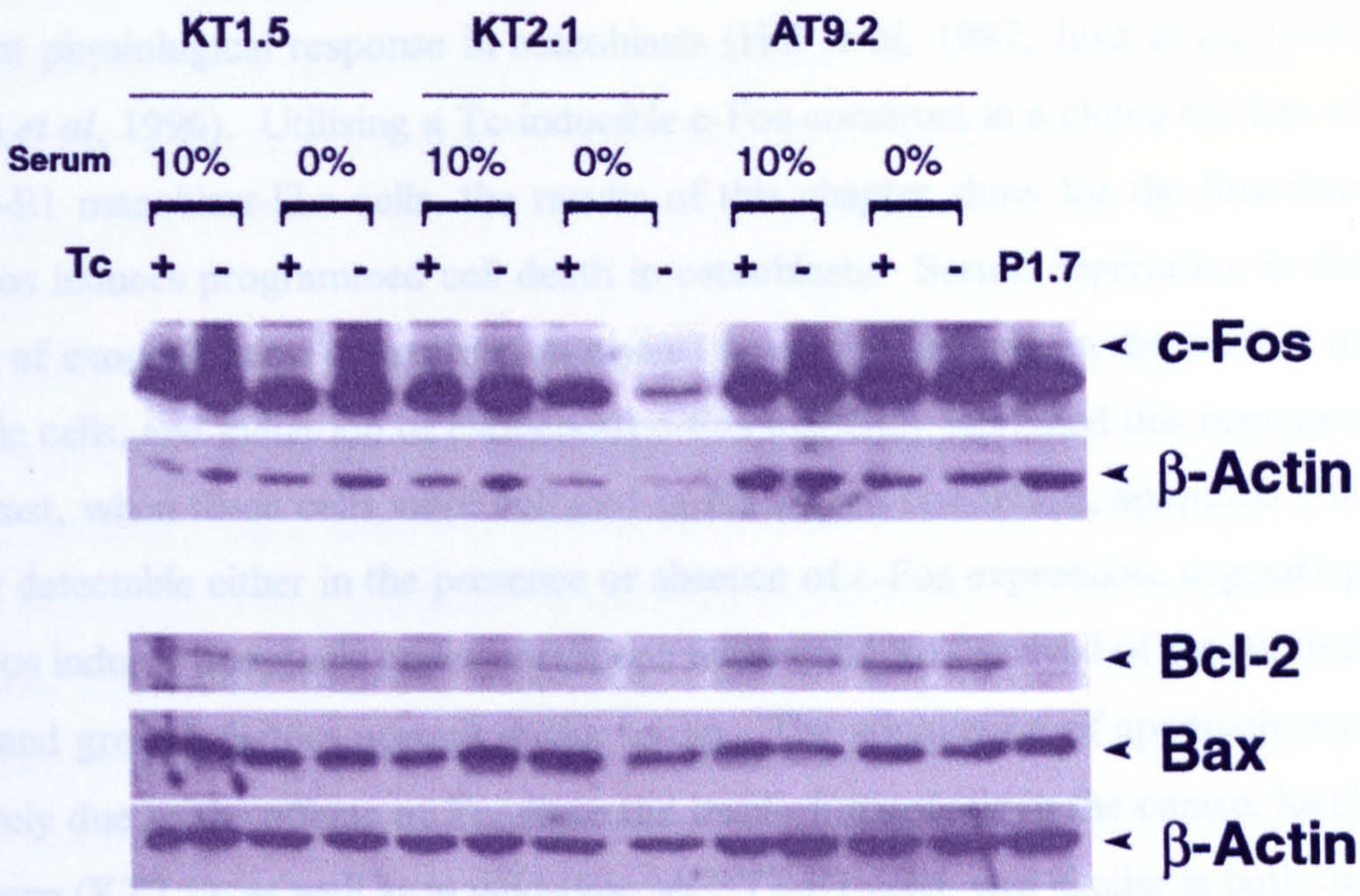


Figure 5.11 – Western blot analysis of Bcl-2, Bax and c-Fos expression in the presence and absence of exogenous c-Fos in KT1.5 cells. Cells (KT1.5, KT2.1, and AT9.2) were cultured ± Tc for 48 hours to induce c-Fos expression, followed by either 0% or 10% serum (± Tc) for 3 days. Western blots were probed with specific antibodies to c-Fos, β-Actin, Bcl-2 and Bax as described in section 2.9. The P1.7 osteoblast like cell line, derived from the bone tumours of H2-c-*fos*LTR mice (Grigoriadis *et al.*, 1993), was used as a positive control.

5.3 Discussion

A number of *in vitro* and *in vivo* studies have demonstrated that apoptosis is an important physiological response in osteoblasts (Hill *et al*, 1997; Jilka *et al*, 1998; Kitajima *et al*, 1996). Utilising a Tc-inducible c-Fos construct in a clonal cell line of MC3T3-E1 osteoblast-like cells, the results of this chapter show for the first time that c-Fos induces programmed cell death in osteoblasts. Serum deprivation in the absence of exogenous c-Fos expression caused a marked increase in the number of apoptotic cells, and induction of exogenous c-Fos markedly increased this response. In contrast, when these cells were cultured in full serum conditions, apoptosis was scarcely detectable either in the presence or absence of c-Fos expression, suggesting that c-Fos induces apoptosis under conditions when cells are deprived of the survival signals and growth factors present in the serum. The stimulation of apoptosis was not merely due to the effects of Tc, since the level of apoptosis in the control luciferase clone (KT2.1), as well as in wild-type MC3T3-E1 cells, was similar in both the presence or absence of Tc. The differences in maximal apoptosis observed in KT1.5 cells, especially in later experiments, is difficult to interpret, but was not as a result clonal drift since cells were always used from an early passage number.

Previous studies have implicated c-Fos as a mediator of apoptosis: c-*fos* expression has been observed to increase in cells undergoing apoptosis, and overexpression of a c-Fos-oestrogen receptor fusion protein induces apoptosis in colorectal cells (Buttyan *et al*, 1988; Colotta *et al*, 1992; Gonzalez-Martin *et al*, 1992; Smeyne *et al*, 1993; Hafezi *et al*, 1997; Wenzel *et al*, 2000; Grimm *et al*, 2001; Preston *et al*, 1996). In contrast, some studies have shown that in certain contexts, c-Fos can protect against apoptosis (Walker *et al*, 1993; Schreiber *et al*, 1995; He *et al*, 1998; Schreiber *et al*, 1999). Experiments using c-*fos* knockout mice have also yielded conflicting results. While some studies have demonstrated that c-*fos* is not required for apoptosis in normal mouse development, others have shown that it is essential for light-induced photoreceptor apoptosis in the retina, an effect which could be rescued by expression of Fra-1 (Roffler-Tarlov *et al*, 1996; Gajate *et al*, 1996; Hafezi *et al*, 1997; Fleischmann *et al*, 2000). Thus, the effects of c-Fos on apoptosis are clearly not equivalent in all systems; rather, it appears that c-Fos affects programmed cell death in a cell- and tissue-specific manner. The data presented in this chapter using MC3T3-E1 subclones demonstrate that c-Fos is an inducer of apopto-

sis in osteoblasts *in vitro*. As explained in section 1.6.5 and 1.6.2, c-Fos is expressed during apoptosis in ossifying regions in late embryonic development and in growth regions during endochondral ossification (Smeyne *et al*, 1993). The physiological relevance of the c-Fos-induced apoptosis in osteoblasts *in vivo* compared with that observed in KT1.5 cells needs to be investigated. Such experiments could compare apoptosis of cells derived from calvaria of c-Fos deficient mice with those of wild type mice.

To study further the mechanisms of how c-Fos sensitises cells to apoptosis, key factors important for apoptotic signalling, such as caspases were investigated. These experiments found that c-Fos may sensitise cells to apoptosis independently of caspase activation. The c-Fos-induced apoptosis could not be blocked using the cell permeable caspase inhibitor, Z-VAD-fmk, although apoptosis induced by serum withdrawal in the absence of c-Fos was partially inhibited. Furthermore, only the highest concentration of DEVD-CHO tested was seen to inhibit c-Fos-induced apoptosis and TNF-α-induced apoptosis in the absence of c-Fos expression, and it remains possible that even higher concentrations may inhibit further. DEVD-CHO is a specific inhibitor of caspase-3, which is an effector caspase rather than an initiator caspase and hence, lies further downstream in the caspase cascade. Overall, this suggests that apoptosis induced by c-Fos may occur independently or downstream of the effector caspases, or at least independently of the caspases that are sensitive to Z-VAD-fmk. Although most apoptotic processes require the activation of caspases as an effector mechanism of apoptosis (reviewed in Hengartner, 2000), many recent studies have reported caspase-independent apoptotic pathways (Maclaren *et al*, 2000; Nunez *et al*, 1998; Daugas *et al*, 2000; McCarthy *et al*, 1997; Susin *et al*, 1999, 2000; Joza *et al*, 2001). Moreover, the presence of a novel caspase-independent death effector, the mitochondrial apoptosis inducing factor (AIF) has been reported (Susin *et al*, 1999, 2000). This factor can stimulate mitochondrial cytochrome c release (Susin *et al*, 1999), in addition to directly inducing chromatin condensation and DNA fragmentation (Daugas *et al*, 2000).

Recently a role for cytochrome c release in c-Myc and v-Jun induced apoptosis has been described (Juin *et al*, 1999; Maclaren *et al*, 2000). This release of cytochrome c was not sufficient to drive apoptosis, but appeared to sensitise the cells to apoptotic stimuli and was independent of caspase activation. Furthermore, McCar-

thy *et al* (1997) reported how Z-VAD-fmk could not inhibit the onset of apoptosis induced by c-Myc but was shown to delay or inhibit the completion of the program once initiated. Therefore, further studies in KT1.5 cells are needed to address whether c-Fos-induced apoptosis involves direct stimulation of cytochrome c release from the mitochondria. In addition, if sustained stimulation of apoptosis is occurring over a 48 hour period, any inhibition of cell death by caspase inhibitors may be by-passed. Furthermore, some caspase inhibitors have been shown to only protect from apoptosis for a certain amount of time, for example DEVD-CHO protection was decreased by 50% after 8hr and for Z-VAD-fmk decreased after 15 hours (Boutillier *et al*, 2000). Clearly further experiments are required to confirm unequivocally whether c-Fos-induced apoptosis in osteoblasts is caspase-dependent or independent. In view of the small window of time for protection, other markers of apoptosis in the presence of caspase inhibitors could be investigated, such as PARP cleavage, cytochrome c release, Annexin V binding and TUNEL.

In KT1.5 cells, Etoposide-induced apoptosis was also shown to be caspase-independent. Previous studies have found a similar result (Robertson *et al*, 2000; Sun *et al*, 1999; Yang *et al*, 1997), although this may also be concentration-dependent: Robertson *et al* (2000) found that whereas high concentrations of Etoposide (50μM) were shown to stimulate apoptosis via induction of cytochrome c, low concentrations (10μM) were inhibited by Z-VAD-fmk. Low concentrations of Etoposide induced apoptosis via a mechanism that involves the release of protein factors that are able to interact with the mitochondria, whereas, higher doses of Etoposide diminish the mitochondrial calcium buffering capacity. Similarly Sun *et al* (1999) showed that Z-VAD-fmk did not inhibit Etoposide-induced cytochrome c release but was able to inhibit caspase-9 activity. The concentration of Etoposide used to induce apoptosis in KT1.5 cells was greater than 50μM and thus it cannot be ruled out that lower concentrations may have been inhibited by Z-VAD-fmk.

It is not uncommon for oncoproteins or transcription factors to have, in addition to their mitogenic and transforming properties, proapoptotic effects. For example, c-Jun, c-Myc, E1A, E2F all stimulate apoptosis in the absence of mitogens (Bossy-Wetzel *et al*, 1997; Shaulian and Karin, 2001; Evan, 1992; Qin *et al*, 1994; Wu and Levine, 1994). c-Myc sensitises cells to a variety of apoptotic signals such as Fas ligand/CD95L and TNF-α, and this is thought to involve the release of cyto-

chrome c from the mitochondria (reviewed by Evan and Littlewood, 1998; Prendergast, 1999). Moreover, c-Jun is a negative regulator of p53 expression/transactivation, and is an important mediator of the pro-apoptotic effects of c-Jun N-terminal kinase (JNK) activation (reviewed by Shaulian and Karin, 2001).

Similarly to c-Myc, v-Jun functions by promoting S phase entry but the cells are unable to accumulate in number in the absence of endogenous growth factors due to apoptotic cell death (Clark and Gillespie, 1997). It has been suggested that c-Fos may promote apoptosis in a similar way to v-Jun (Pandey and Wang, 1995). Indeed, recent work in our laboratory by Sunters *et al* (2000) has shown that induction of ectopic c-Fos in AT9.2 cells stimulates cell cycle entry by inducing cyclin A expression and cyclinA/CDK2 kinase activity. Thus, it could be predicted that enhanced cell cycle progression is responsible for the increased apoptosis observed in this investigation.

Experiments using cell cycle inhibitors clearly showed a link between the c-Fos effects on cell cycle progression and apoptosis. c-Fos-induced apoptosis was reduced dose-dependently by Roscovitine, which is a specific inhibitor of cyclin-dependent kinase (CDK)-2 (Alessi *et al*, 1998; Schutte *et al*, 1997). When many of the cells in the population were arrested in the G_0 stage of cell cycle, the presence of ectopic c-Fos may have been forcing these cells to progress into cell cycle by increasing the available CDK2 in the cell, and thus predisposing them to apoptotic insult. Treatment with Roscovitine was then preventing the group of cells which tried to progress through the cell cycle by directly suppressing any CDK-2 activity.

Apoptosis induced by Etoposide was abrogated completely by treatment of cells with Roscovitine. A number of studies suggest that cells progressing through the cell cycle appear more susceptible to apoptosis than quiescent cells, that is, inappropriate induction of cell cycle progression may cause certain cell cycle effectors to promote apoptosis (reviewed in Evan, 1995; Evan and Littlewood, 1998). Cell cycle checkpoints have been shown to feature in some apoptotic pathways, with apoptotic cells often displaying abnormal levels of cell cycle components or the apoptosis has been induced by deregulation of cell cycle factors (e.g., Meikrantz *et al*, 1994). The stage in the cell cycle most sensitive to apoptosis appears to be between late G_1 or early S phases. Cell cycle arrest in G_0 or early G_1 suppresses apoptosis in response to a range of apoptotic stimuli. In contrast, arrest late in G_1 or in S

phase can accelerate or potentiate apoptosis, thus indicating that factors must be present in late G_1 and S phase whose activities facilitate the execution of apoptosis. *In vivo* apoptosis has been shown to occur primarily in proliferating tissue and is associated with induction of proliferation-associated genes, such as c-*myc* and c-*fos* (reviewed by Meikrantz and Schlegel, 1995).

There are many lines of evidence from diverse systems suggesting that the induction of apoptosis is associated with activation of cyclin A-dependent kinases, i.e. CDK2 (Meikrantz and Schlegel, 1996). Overexpression of cyclin A and CDK2 increased apoptosis in cells in which the *Bcl-2* gene is overexpressed (*Bcl-2*$^{+}$), that is, these factors can override the anti-apoptotic properties of Bcl-2 (Meikrantz and Schlegel, 1996). Gil-Gomez *et al* (1998) proposed that p53 and Bcl-2/Bax regulation was upstream of CDK2 activation in thymocyte apoptosis and that CDK2 activation was the crucial point at which cell cycle and cell death pathways interact. Furthermore, caspase-3 has been shown to cleave or inactivate the CDK inhibitors, p21 and p27, in cells undergoing apoptosis, leading to increased CDK2 activity in the early stages of apoptosis (Levkau *et al*, 1998; Jin *et al*, 2000). Thus, there are many lines of evidence implicating cyclin A and CDK2 as key players in apoptosis.

Research by Harvey *et al* (1998) showed that changes in CDK activity during apoptosis are also downstream of effector caspases within a sequential and linear pathway. If CDK activation is downstream of caspase cascades, this helps to support a caspase-independent mechanism for inducing apoptosis in KT1.5 cells, such that c-Fos is increasing CDK activity which then has the ability to induce apoptosis when normal cell cycle control is being deregulated. By inappropriately bypassing mitotic checkpoints, such as in c-Fos-induced KT1.5 cells, the deregulated CDK activity may then result in stimulating cell death. Alternatively, these CDKs may proceed to phosphorylate and activate other apoptotic proteins such as nucleases that are involved in apoptosis, or may alter nuclear envelope structure to allow damaging agents into the nucleus (King and Cidlowski, 1995).

Further evidence that the cell cycle may be involved in c-Fos induced apoptosis, comes from the observation that ectopic expression of the CDK inhibitor $p21^{WAF1,CIP1,SDI1}$ reduced the c-Fos-induced apoptosis. Besides its well-characterised role as an inhibitor of CDK activity and cell cycle progression, p21 has also been shown to prevent apoptosis in various cells. For example, p21 has been shown to

mediate the anti-apoptotic effects of NF-κB and can bind to ASK1 (apoptosis signal related kinase 1) to inhibit apoptosis (e.g., Wang and Walsh, 1996; Lu *et al*, 1998; Javelaud *et al*, 2000). The mechanisms through which p21 inhibits c-Fos-induced apoptosis in KT1.5 cells remains to be elucidated. Interestingly, while there are some parallels between c-*fos* and c-*myc* action, CDKs are not thought to be required for c-Myc-induced apoptosis, which is not inhibited by the CDK inhibitors Roscovitine, p21, p27, and p16 (Rudolph *et al*, 1996). This suggests that c-*fos* and c-*myc* exert their apoptotic effects via different mechanisms.

The apoptosis induced by c-Fos in KT1.5 cells was also inhibited by ectopic expression of *Bcl-2*. This suggests that the cells are undergoing a *bona fide* apoptosis following c-Fos-induction. The overexpression of *Bcl-2* proto-oncogene has been shown to inhibit apoptosis in a number of different cell types including apoptosis induced by c-*fos* expression (reviewed in Reed, 1992; Korsmeyer, 1992; Preston *et al,* 1996). Indeed, the survival effects exerted by growth factors and cytokines are in many instances mediated by regulation of endogenous *Bcl-2* levels (Oltvai *et al*, 1993; Hoffman and Liebermann, 1998). The rescue of apoptosis by transfected *bcl-2* suggested that the expression of Bcl-2 family members may be altered in the presence of c-Fos. However, Bcl-2 and Bax protein levels were unaltered in the presence or absence of exogenous c-Fos in all cell lines tested, suggesting that these genes were not directly regulated by c-Fos. It remains possible, however, that the expression of other members of the Bcl-2 family, for example, Bcl-xl and Bak may be altered.

The mechanism of action of Bcl-2 is quite complex and not entirely understood, with many functions for Bcl-2 being postulated (see e.g., review by Tsujimoto and Shimizu, 2000). Of most relevance to this thesis, is the fact that the inhibition of apoptosis by Bcl-2 has also been linked to a reduction in cell cycle progression, in particular, in the prolongation of the G_1 phase of the cell cycle (Meikrantz and Schlegel, 1996; Borner, 1996; Mazel *et al*, 1996; Philips *et al*, 1999; Vairo *et al*, 2000). Thus, it cannot be ruled out that the inhibition of apoptosis by ectopic Bcl-2 expression in KT1.5 cells may also be mediated through cell cycle mechanisms.

5.4 Conclusions

Inducible overexpression of c-Fos in osteoblastic cells resulted in enhanced apoptosis induced by different apoptotic stimuli. Using specific inhibitors, this appeared to be independent of caspase activation but it did involve the cell cycle machinery. Taken together with previous work from our laboratory that c-Fos additionally increases cyclin A/CDK2 activity in these cells, the data presented provide a novel link between cell cycle control and programmed cell death in osteoblasts.

6. The role of *Msx* genes in osteoblast function

6.1 Introduction

The *Msx* homeoproteins, Msx1 and Msx2, have been shown to have a role in the development of mineralising tissues in craniofacial bones and teeth (Takahashi *et al*, 1991; Jabs *et al*, 1993; MacKenzie *et al*, 1992; Jowett *et al*, 1993; see section 1.7.1); where they act as transcriptional repressors of gene function in embryonic development. Furthermore, *Msx2* may play a role in the differentiation of osteoprogenitor cells, preventing differentiation and promoting proliferation of cells specifically at the osteogenic fronts of calvariae (Liu *et al*, 1995; Liu *et al*, 1999; see Chapter 1.7.1.1). Evidence is emerging of upstream regulatory processes of *Msx* genes. For example, BMPs (BMP-2, BMP-4 and BMP-7), FGFs, oestrogen, and retinoic acid have all been shown to induce *ex vivo* and *in vitro Msx* expression in various cellular systems (e.g., Bei and Maas, 1998; Lee *et al*, 1999; Wang *et al*, 1998; Kim *et al*, 1998; Tucker *et al*, 1998; Kettunen *et al*, 1998; Phippard *et al*, 1996; Abud *et al*, 1996; Wang and Sassoon, 1995; Sirard *et al*, 2000).

Recent studies have shown that transcription factors, such as Cbfa1, which are expressed in early development may also have a role in post-natal bone formation (Ducy *et al*, 1999). More specifically, Hodgkinson *et al* (1993) demonstrated a role for *MSX2* in adult osteoblastic bone formation and its regulation of expression *in vitro* by 1,25-$(OH)_2D_3$. However, no vitamin D-response elements were found in DNA regulatory sequences up to 1.2kb upstream of *Msx2*, suggesting that the effects of 1,25-$(OH)_2D_3$ on *Msx2* expression may be mediated indirectly through other mechanisms. Parathyroid Hormone (PTH) is another osteotropic hormone, which acts in conjunction with 1,25-$(OH)_2D_3$ to co-ordinate the regulation of bone metabolism and calcium homeostasis (see section 1.3.3.2), and which may be involved in this signalling pathway. Similar to *Msx2*, PTH can also inhibit osteoblast differentiation and mineralisation, although these effects are dependent on the stage of differentiation and duration of administration (Dempster *et al*, 1993; Isogai et al, 1996; see section 1.3.3.2).

This study investigated whether Msx2 was involved in the signalling pathways for PTH in osteoblasts. This involved determining the regulation of both endogenous *Msx2* gene expression, as well as *Msx2* promoter activity by different hormones and growth factors, such as PTH in different osteoblastic cell lines. In addition, this

study explore further the *in vivo* expression of *Msx* genes in adult cells of the osteoblast lineage using *in situ* hybridisation analysis of mouse bone and in particular, by utilising *Msx1-lacZ* knock-in transgenic mice.

6.2 Endogenous *Msx2* expression in osteoblast-like cell lines following treatment with PTH

To evaluate the endogenous expression of *Msx2* in osteoblasts, Northern blot analysis was conducted on mRNA from SaOS-2 cells in culture, following treatment with PTH (1-34).

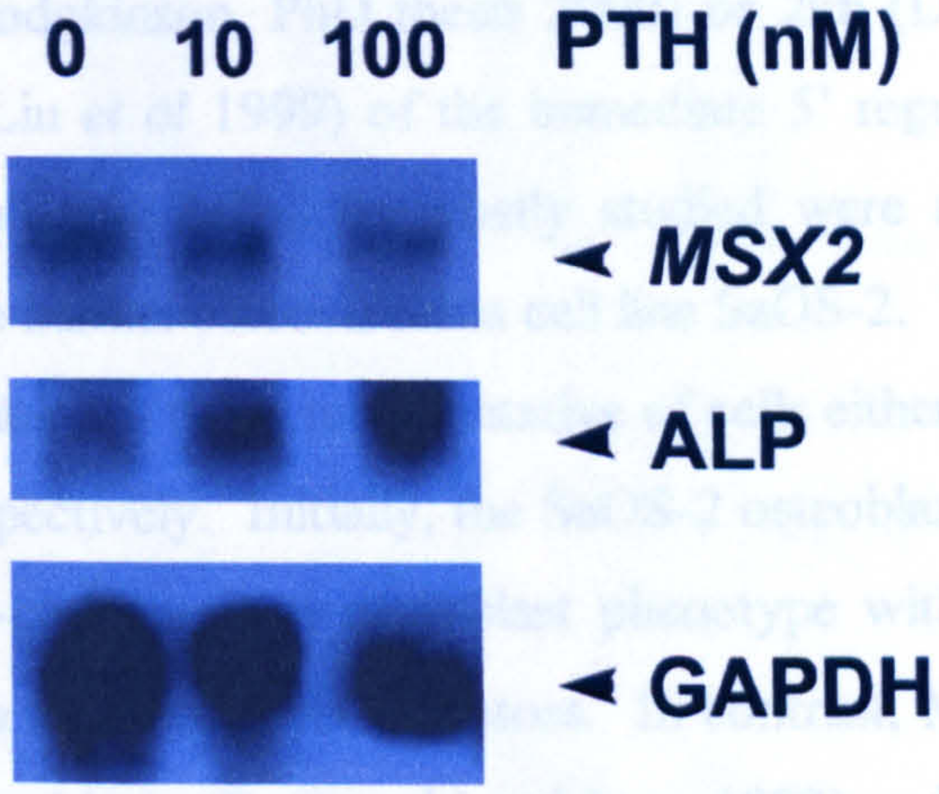


Figure 6.1 - Northern Blot analysis of *MSX2* expression in SaOS-2 cells and regulation by PTH. SaOS-2 cells were treated for 24 hours with PTH or vehicle control before extraction of poly $(A)^+$ RNA and Northern blot analysis. A radiolabelled probe containing *Msx2* coding sequence was used to probe the membrane (see section 2.8.4). GAPDH transcripts were probed to control for RNA loading. Expression of alkaline phosphatase (ALP) was probed to assess for PTH functionality in these cells. Cells were also treated with 10 and 100nM PTH for shorter periods (2 and 4 hours) but similar results were found (data not shown).

SaOS-2 cells were shown to express basal levels of *MSX2*. Furthermore, treatment of SaOS-2 cells with PTH showed that *MSX2* was not regulated by PTH under the conditions used when standardised for GAPDH expression (Figure 6.1). The regulation of alkaline phosphatase was used as a positive control for PTH activity and demonstrated a small induction of alkaline phosphatase expression following treatment with PTH (Figure 6.1). These results suggest that after 24 hour treatment, PTH has no effect on *Msx2* expression.

6.3 The effects of osteotropic factors on the regulation of 1.2 and 2kb *Msx2* reporter gene constructs in osteoblast-like cell lines

Factors known to be important in bone development, in particular PTH, were screened for their ability to induce the expression of two murine *Msx2*-CAT (Chloramphenicol acetyltransferase) reporter gene constructs. These constructs consisted of either 1.2kb (Jane Hodgkinson, PhD thesis 2000) or 2kb (Dr R. Maxson, *pers comm*; Liu *et al* 1994; Liu *et al* 1999) of the immediate 5' regulatory sequence of *Msx2* gene. The osteoblastic cell lines mostly studied were murine osteoblastic MC3T3-E1 cells and the human osteosarcoma cell line SaOS-2. These were used to compare the regulation in cells types representative of cells either early or late in the osteoblastic lineage, respectively. Initially, the SaOS-2 osteoblast cell line was chosen because of its well-characterised osteoblast phenotype with respect to its response to PTH and high levels of PTH receptors. In contrast, MC3T3-E1 cells are representative of preosteoblasts (Franceschi and Iyer, 1992) and were selected as a model for an earlier stage of osteoblast maturation.

For SaOS-2 cells, the conditions for transfection with Lipofectamine were initially optimised as described in section 2.6.6.2. Following this, cells were transfected with reporter constructs before treatment with a suitable range of concentrations of PTH, 1,25-$(OH)_2D_3$, BMP-2, BMP-4, FGF-4, FGF-8, Sonic hedgehog and dexamethasone. These are factors known to be important in bone development and to influence the differentiation of osteoblasts. With the exception of PTH, cells were treated with the factors for 24 hours; treatment times for PTH were 1, 3, 6, 24, and 48 hours. Cell extracts were then prepared for assessment of CAT activity.

6.3.1 The effect of PTH on *Msx2*-CAT reporter gene activity

SaOS-2 and MC3T3-E1 cells were transfected with *Msx2*-CAT reporter constructs and treated for various time points with a range of concentrations of PTH. PTH appeared to show no significant induction of reporter gene activity in the cell lines studied. Figure 6.2 shows the data summarised from at least three or more experiments whereby cells were treated with 100nM PTH; similar results were observed with 48 hour treatment with PTH and for lower concentrations of PTH, but have not been shown in Figure 6.2.

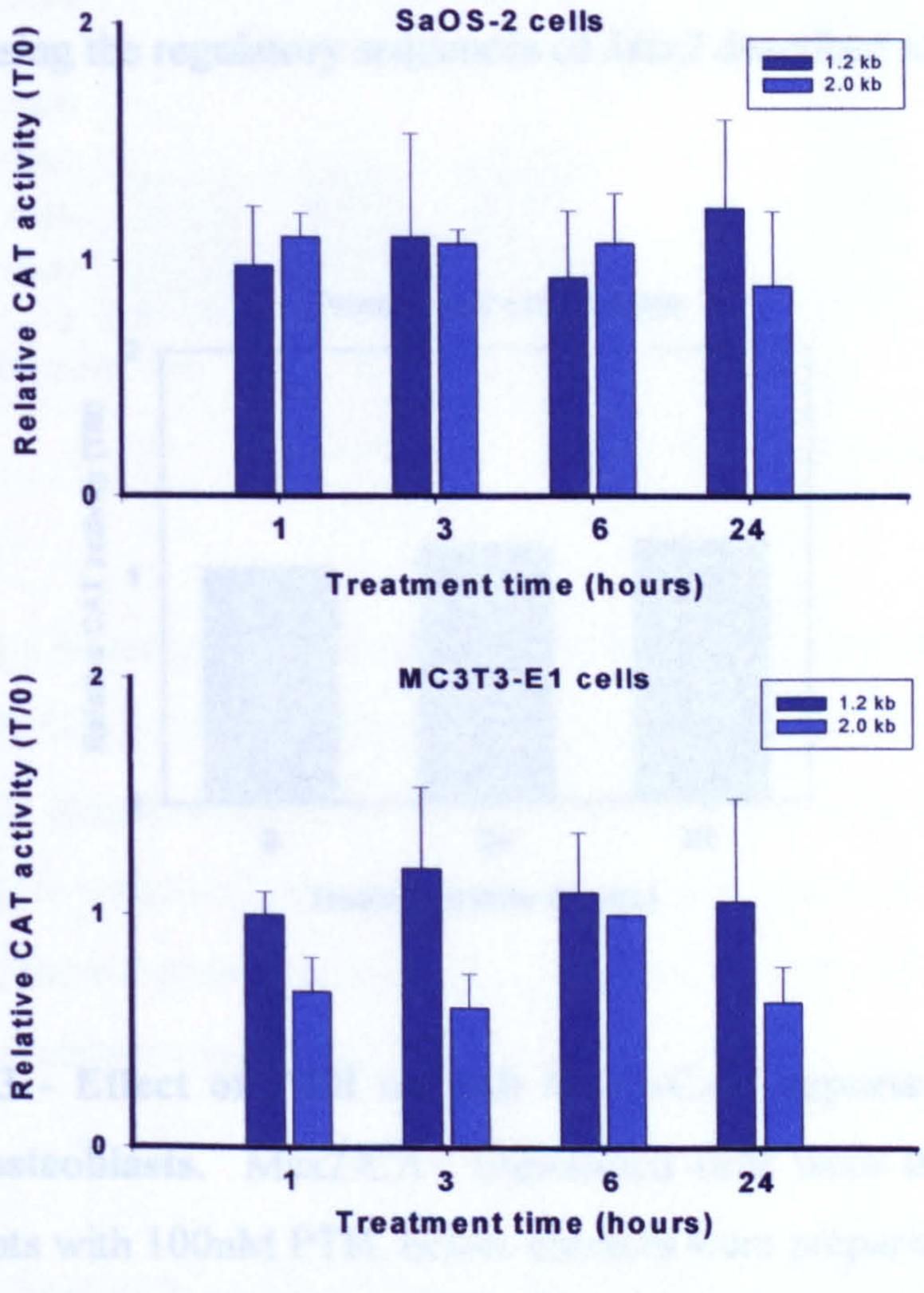


Figure 6.2 - Effect of PTH on Msx2-CAT reporter gene activity in SaOS-2 and MC3T3-E1 cells. *Msx2*-CAT transfected cells were treated for the indicated time points with 100nM PTH, before extracts were prepared for assessment of CAT activity. Data represents mean relative CAT activity (treatment/control values) ± S.D. from three or more experiments. The counts per minute per μg protein extract were expressed as a ratio of treatment/control (T/0).

Following these experiments, additional cell lines were studied to determine whether the lack of regulation by PTH with these reporter constructs in SaOS-2 and MC3T3-E1 cells was a specific characteristic of the phenotype of these cells. Similar results were obtained using rat ROS 17/2.8 osteoblasts, human MG63 osteoblastic cells and non-osteoblastic COS cells (data not shown). Furthermore, primary rat calvarial osteoblasts were transfected with the 2kb *Msx2*-CAT construct and treated with PTH in one experiment. However, under these conditions, there was no stimulatory activity of the reporter gene construct with PTH when compared to vehicle control (Figure 6.3). Overall, these results suggest that *Msx2* is not regulated by PTH using the regulatory sequences of *Msx2* described above and in the cell lines studied.

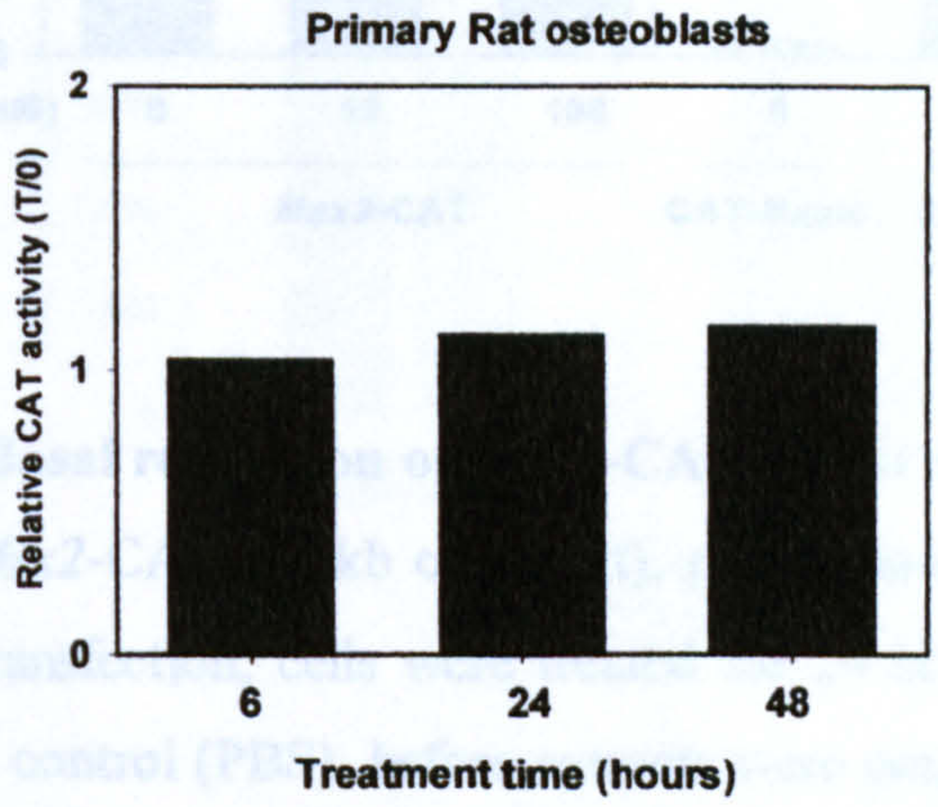


Figure 6.3 - Effect of PTH on 2kb Msx2-CAT reporter gene activity in primary rat osteoblasts. Msx2-CAT transfected cells were treated for the indicated time points with 100nM PTH, before extracts were prepared for assessment of CAT activity. Data represents mean relative CAT activity (treatment/control values) ± S.D. from one experiment. The counts per minute per μg protein extract were expressed as a ratio of treatment/control (T/0).

The SV40-CAT vector was used as positive control for CAT activity in all experiments. In the cell lines studied, there was a strong regulation of SV40-CAT vector as determined by CAT activity, which was between 1.5 and 2.4-fold greater

than *Msx2*-CAT vectors in most experiments (Figure 6.4). Interestingly, there was a basal expression of the *Msx2*-CAT vectors in all of the osteoblastic cell lines assessed, which was significantly greater than that found with the pCAT-Basic vector alone (Figure 6.4).

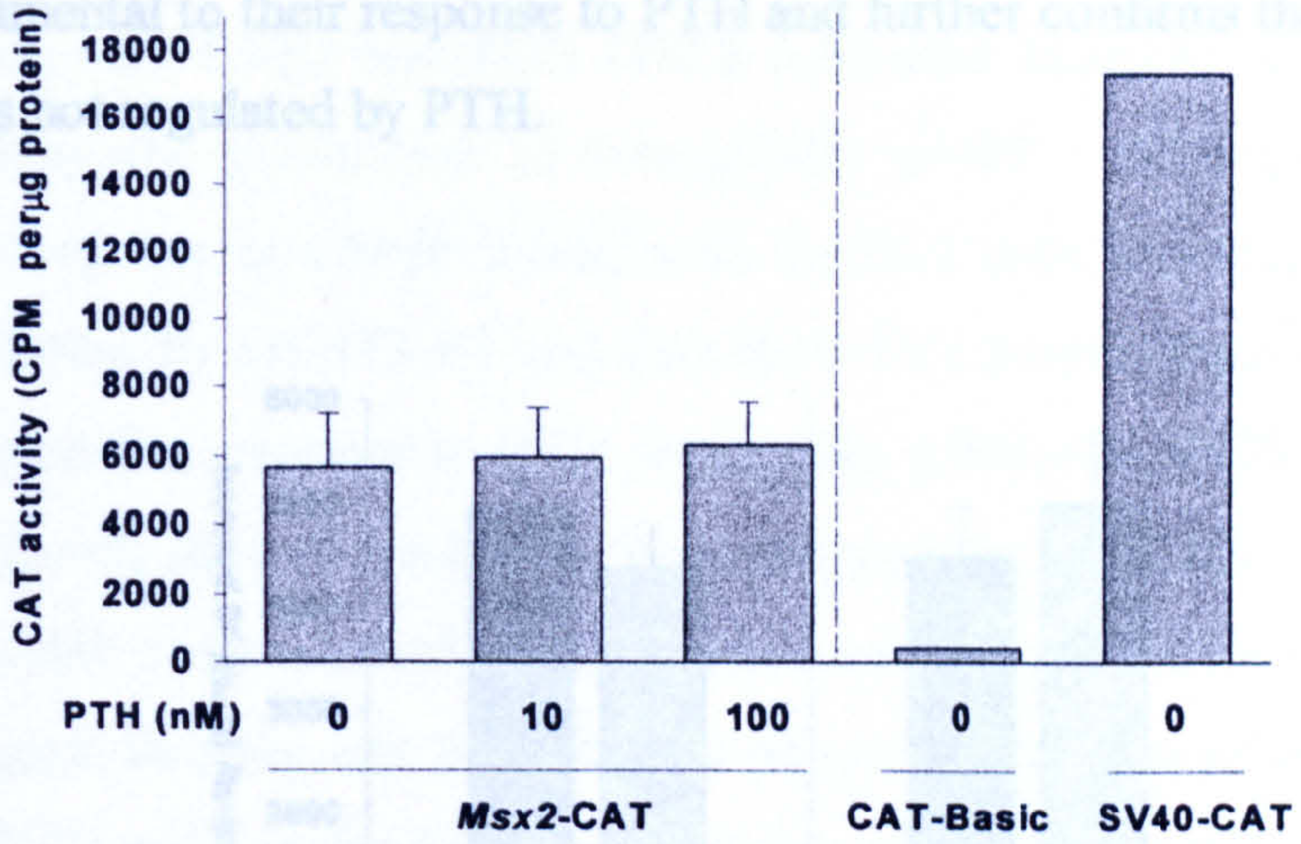


Figure 6.4 - Basal regulation of *Msx2*-CAT vector in SaOS-2. Cells were transfected with *Msx2*-CAT (1.2kb construct), pCAT-Basic or pSV40-CAT vectors. Following transfection, cells were treated for 24 hours with PTH (10 and 100nM) or vehicle control (PBS), before extracts were prepared for assessment of CAT activity. Data represents mean (± S.D.) CAT activity of triplicate samples from one representative experiment.

Co-transfecting cells with constructs, such as CMV-SPAP, can result in a lower transfection efficiency and reporter gene response as a result of the plasmid of interest having to compete with the co-transfected DNA. Therefore, a comparison was made between *Msx2*-CAT-transfected and CMV-SPAP/*Msx2*-CAT co-transfected SaOS-2 cells in their response to PTH. This was done to confirm that the lack of response to PTH that was observed in SaOS-2 cells was not as a result of less DNA entering the cell and hence being available for modulation by mediators of PTH signalling. SaOS-2 cells were transfected with *Msx2*-CAT (1.2kb construct) alone

or *Msx2*-CAT plus CMV-SPAP vector. Following transfection, cells were treated for 24 hours with PTH, before extracts were prepared for assessment of CAT activity.

The response of transfected SaOS-2 cells to PTH was the same for cells transfected with *Msx2*-CAT alone as that for CMV-SPAP/*Msx2*-CAT co-transfected cells, with no significant regulation of *Msx2*-CAT activity for either group (Figure 6.5). This confirms that co-transfecting the cells does not affect them in such a way that is detrimental to their response to PTH and further confirms that the *Msx2*-CAT construct is not regulated by PTH.

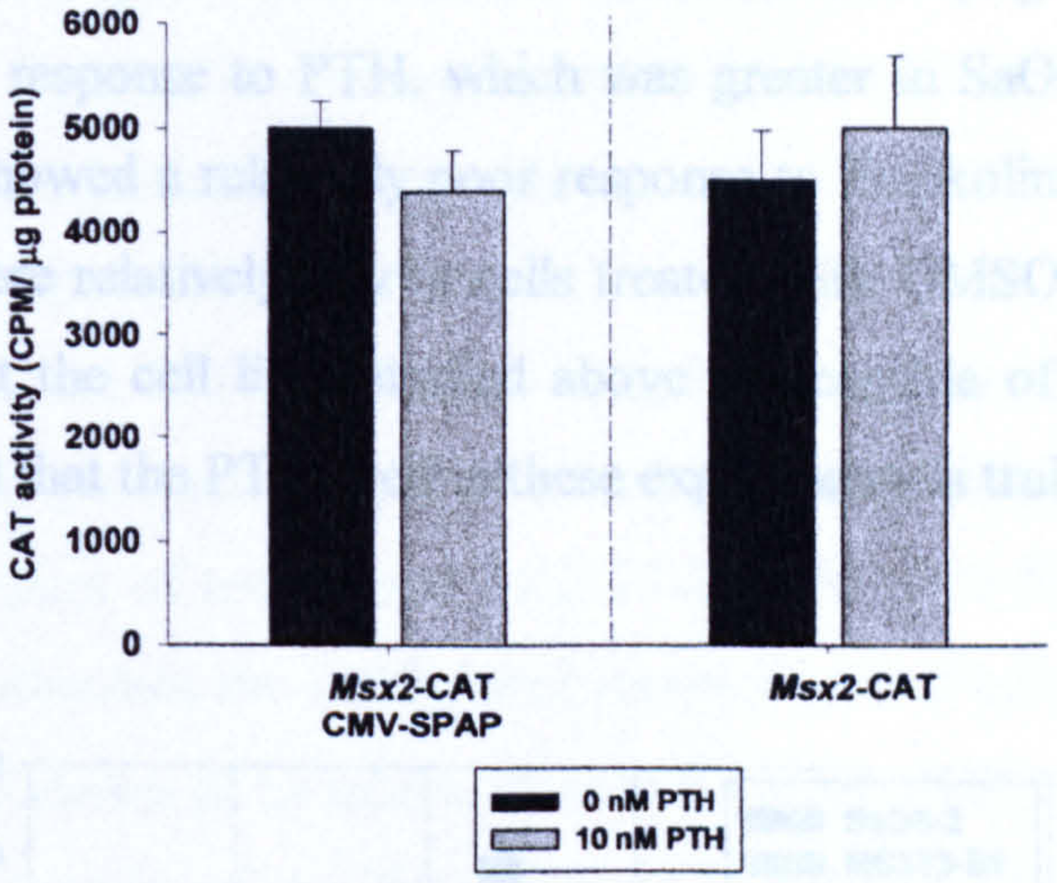


Figure 6.5 - Comparative response of SaOS-2 cells to PTH in *Msx2*-CAT-transfected and co-transfected cells. Cells were transfected with *Msx2*-CAT (1.2kb construct) alone or *Msx2*-CAT plus CMV-SPAP vector. Following transfection, cells were treated for 24 hours with PTH (0 and 100nM), before extracts were prepared for assessment of CAT activity. Data represents mean (± S.D.) CAT activity (treatment/control values) of triplicate samples from one representative experiment.

6.3.2 Determination of the functionality of PTH in SaOS-2, MC3T3-E1 and ROS 17/2.8 cells

The action of PTH is mediated by the peptide binding to its membrane-bound receptor and subsequent activation of adenylate cyclase. This enzyme stimulates the increase in levels of cAMP, thereby stimulating intracellular signalling cascades. To confirm whether PTH is functional in the cell lines under study, its ability to stimulate cAMP was assessed.

SaOS-2, MC3T3-E1 and ROS 17/2.8 cells were found to respond to PTH as determined by the production of intracellular cAMP. PTH produced a dose-dependent response in cAMP levels, with SaOS-2 cells showing the greatest response, followed by MC3T3-E1 and then ROS 17/2.8 cells (Figure 6.6). Forskolin produced a similar response to PTH, which was greater in SaOS-2 cells; although ROS 17/2.8 cells showed a relatively poor response to Forskolin. As expected, the levels of cAMP were relatively low in cells treated with DMSO or IBMX. These results suggest that the cell lines studied above are capable of responding to the actions of PTH and that the PTH used in these experiments is truly functional.

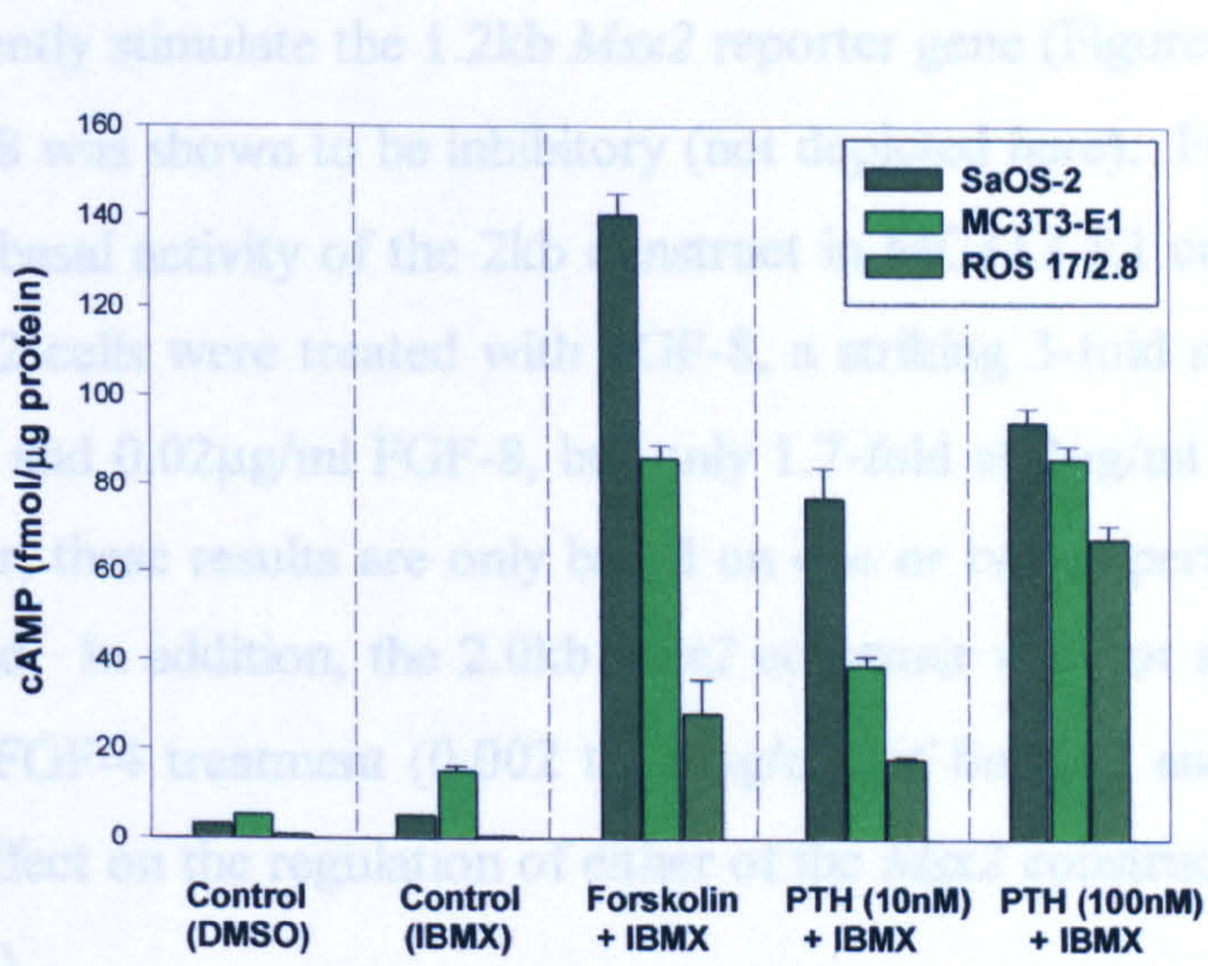


Figure 6.6 - PTH stimulation of cAMP production in SaOS-2, MC3T3-E1 and ROS 17/2.8 cells. SaOS-2, MC3T3-E1 and ROS 17/2.8 cells were treated as indicated and cAMP was extracted and analysed as described in section 2.6.12. Data represents mean (± S.D.) of triplicate samples from one experiment.

6.3.3 Regulation of *Msx2* reporter gene constructs by other factors in bone development

To confirm that the *Msx2*-CAT constructs could be regulated by other factors known to be important in osteoblast function, and reportedly the regulation of *Msx2*, SaOS-2 and MC3T3-E1 cells were transfected with *Msx2*-CAT constructs and treated with BMPs, FGFs, 1,25-$(OH)_2D_3$, dexamethasone and Sonic hedgehog.

The treatment of SaOS-2 cells with BMP-2 showed a marked 4 to 5-fold induction of the 2.0kb *Msx2* reporter gene activity and a lesser 2-fold induction of the 1.2kb construct (Figure 6.7 A). Similarly, BMP4 demonstrated stimulatory activity of the 1.2kb *Msx2* reporter gene in SaOS-2 cells, with a 3.6-fold induction occurring at 20 μg/ml BMP-4 (Figure 6.7 B). There was only a marginal 1.5-fold stimulation of 1.2kb construct by BMP-4 observed in MC3T3-E1 cells (Figure 6.7 B). There was also a lack of stimulation of the 2kb construct in MC3T3-E1 cells by both BMP2 and BMP-4 (data not shown).

The growth factors FGF-8 and Sonic hedgehog (Shh), were shown to stimulate *Msx2*-CAT reporter gene activity but to a lesser extent than the induction observed with BMPs. Treatment of transfected MC3T3-E1 cells with FGF-8 was shown to dose-dependently stimulate the 1.2kb *Msx2* reporter gene (Figure 6.7 C). However, 2μg/ml FGF-8 was shown to be inhibitory (not depicted here). Furthermore, FGF-8 inhibited the basal activity of the 2kb construct in MC3T3-E1 cells (Figure 6.7 C). When SaOS-2 cells were treated with FGF-8, a striking 3-fold stimulation was observed at 0.2 and 0.02μg/ml FGF-8, but only 1.7-fold at 2μg/ml FGF-8 (Figure 6.7 D). However, these results are only based on one or two experiments and need to be reproduced. In addition, the 2.0kb *Msx2* construct was not studied under these conditions. FGF-4 treatment (0.002 to 2 μg/ml) of SaOS-2 and MC3T3-E1 cells showed no effect on the regulation of either of the *Msx2* constructs (data not shown for simplicity).

SaOS-2 cells transfected with either the 1.2 or 2.0kb *Msx2* constructs were also treated with Shh. Shh (5 μg/ml) produced a 1.6-fold stimulation of 1.2kb *Msx2* reporter gene activity (Figure 6.7 E) and a 2-fold stimulation of 2.0kb reporter gene activity for all concentrations of Shh. Once again these results are based on preliminary work, consisting of only one experiment, and the regulation of *Msx2* by Shh in MC3T3-E1 cells needs to be investigated.

MSX2 expression has previously been shown to be induced by 1,25-$(OH)_2D_3$ in normal human bone cells (Hodgkinson *et al*, 1993). However, no significant induction of *Msx2*-CAT reporter gene activity was observed when SaOS-2 cells were treated with 1,25-$(OH)_2D_3$ following transfection with either the 1.2 or 2.0kb *Msx2* constructs (Figure 6.7 F). Similarly, when MC3T3-E1 cells were treated with 1,25-$(OH)_2D_3$ there was no induction of reporter gene activity above basal levels (data not shown). SaOS-2 cells were also treated with dexamethasone, which subsequently reduced the regulation of the 2.0kb *Msx2*-CAT reporter gene activity; but had no effect on the 1.2kb sequence (Figure 6.7 F). Interestingly, this inhibition of reporter gene activity by dexamethasone was also observed for MC3T3-E1 (data not shown).

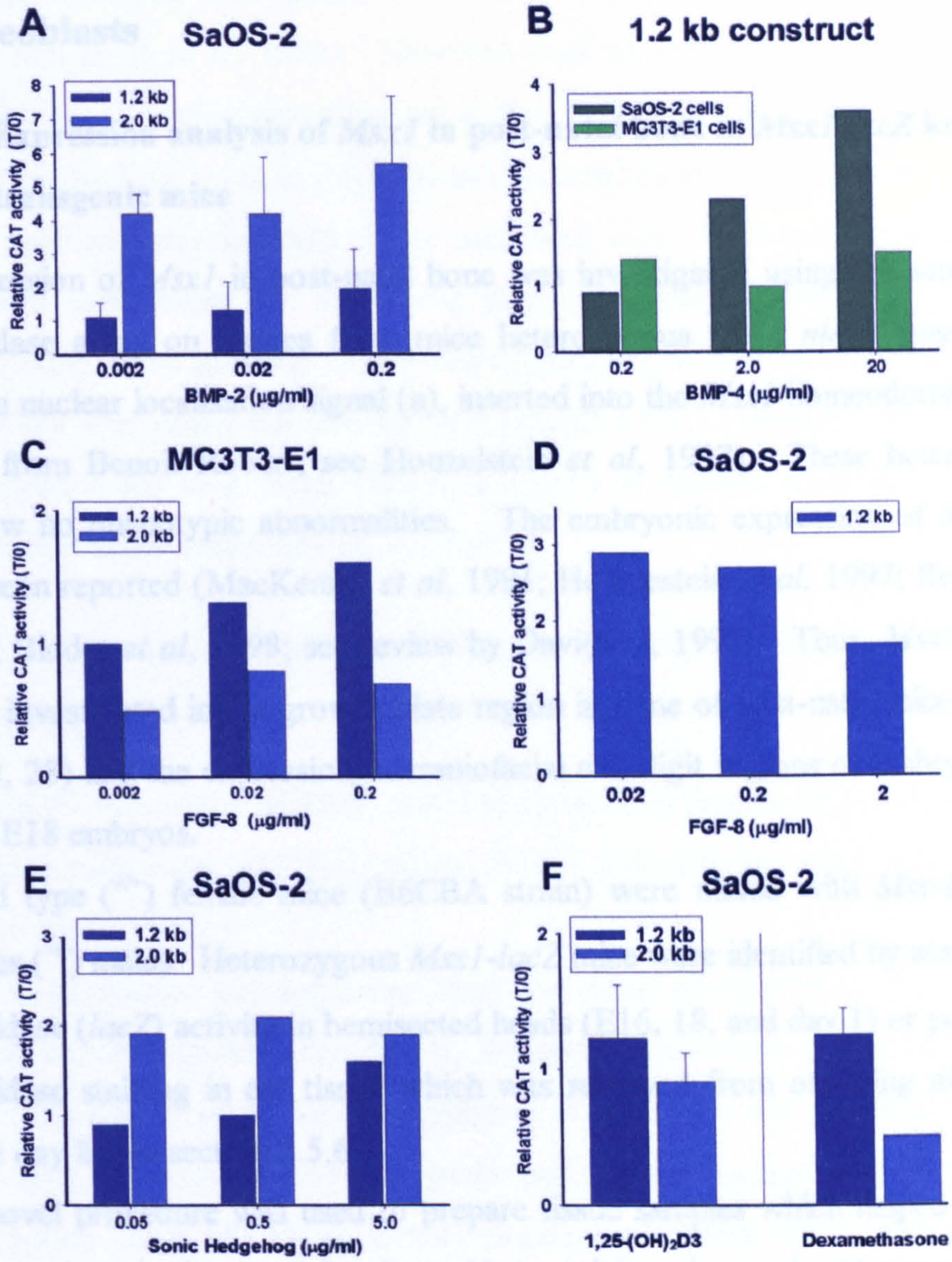


Figure 6.7 – Effect of osteotropic growth factors and hormones on *Msx2*-CAT reporter gene activity in SaOS-2 and MC3T3-E1 cells. *Msx2*-CAT transfected cells were treated at the indicated concentrations with BMP-2, BMP-4, FGF-8, Shh, 1,25-$(OH)_2D_3$, dexamethasone for 24 hours, before extracts were prepared for assessment of CAT activity. Data represents mean relative CAT activity (treatment/control values) ± S.D. from three experiments. The counts per minute per µg protein extract were expressed as a ratio of treatment/control (T/0). Data is representative of at least three experiments except for data denoted by an absent error bar.

6.4 Investigation into the expression of *Msx* genes in adult murine osteoblasts

6.4.1 Expression analysis of *Msx1* in post-natal bone in *Msx1-lacZ* knock-in transgenic mice

The expression of *Msx1* in post-natal bone was investigated using the sensitive β-galactosidase assay on tissues from mice heterozygous for a *nlacZ* gene, which contains a nuclear localisation signal (n), inserted into the *Msx1* homeodomain (mice supplied from Benoit Robert; see Houzelstein *et al*, 1997). These heterozygous mice show no phenotypic abnormalities. The embryonic expression of *Msx1* has already been reported (MacKenzie *et al*, 1991; Houzlestein *et al*, 1997; Reginelli *et al*, 1995; Bidder *et al*, 1998; see review by Davidson, 1996). Thus, *Msx1* expression was investigated in the growth plate region in bone of post-natal mice (days 1, 8, 15, 22, 28) and the expression in craniofacial and digit regions of embryonic (E) E16 and E18 embryos.

Wild type ($^{+/+}$) female mice (B6CBA strain) were mated with *Msx-lacZ* heterozygous ($^{+/-}$) males. Heterozygous *Msx1-lacZ* mice were identified by assessing β-galactosidase (*lacZ*) activity in hemisected heads (E16, 18, and day 1) or positive β-galactosidase staining in ear tissue which was removed from offspring after post-natal age day 8 (see section 2.5.6).

A novel procedure was used to prepare tissue samples which helped to eliminate unnecessary background β-galactosidase staining observed with neonatal samples when using this histochemical method, or as a result of demineralisation (see section 2.5.6). Briefly, tissue was fixed (paraformaldehyde; 4%), demineralised (formic acid/sodium citrate) and cryoprotected (30% sucrose), before freezing at -80°C. Cryostat sections (5-7μm) were fixed and stained for β-galactosidase activity in X-gal solution and counter-stained using safranin. Adjacent sections were stained with haematoxylin and eosin.

In foetal tissue, *lacZ* expression was observed at high levels in developing tooth germs, hair follicles, ossifying regions of the mandible, maxilla and cranium, and perichondrial regions surrounding the developing digits (Figure 6.8 B, D, F). No β-galactosidase staining was observed in sections taken from wild-type bone (data not

shown). This expression correlates with the fundamental role for which Msx1 plays in craniofacial skeletal development and limb formation (Robert *et al*, 1989; Hill *et al*, 1089; McKenzie *et al*, 1991). However, *lacZ* expression was only detected at day 1 in post-natal tissue; investigations at day 8 showed that *lacZ* expression had decreased by this time point in the post-natal skeletal tissue studied (Figure 6.9). At day 1 of post-natal development, *Msx1* continued to be expressed in the perichondrial regions surrounding the developing digits and specific osteoblasts lining the trabecular bone of the growing long bones (Figure 6.9 A, B). *Msx1* expression was absent by day 8 of post-natal development (figure 6.9 C). Similarly no expression was observed in the long bones (Figure 6.9 E-F) or calvaria (data not shown) of mice from post-natal stages day 15, 22, and 28.

6.5 *In situ* analysis of *Msx* gene expression during bone development

To confirm the expression of *Msx1*, and that of *Msx2*, in post-natal bone tissue, non-transgenic mouse bone was analysed by *in situ* hybridisation using specific riboprobes for these genes.

Radioactive sense and anti-sense *Msx1* and *Msx2* riboprobes were synthesised using murine cDNA probes according to the method described in section 2.3.5 and 2.10.1.2 (MacKenzie *et al*, 1991; Monaghan *et al*, 1991) and radioactive *in situ* hybridisation (see section 2.10.3) was performed on bone sections from hemi-sected heads and long bones of CD1 mice representative of various stages of bone development. Late stage embryonic (stage E17) and early post-natal (day 3) sections were included as a positive control for expression analysis based on results previously reported (Monaghan *et al*, 1991; Reginelli, *et al*, 1995; Bidder *et al*, 1998). Following hybridisation, sections were counterstained with haematoxylin and eosin, mounted and photographed.

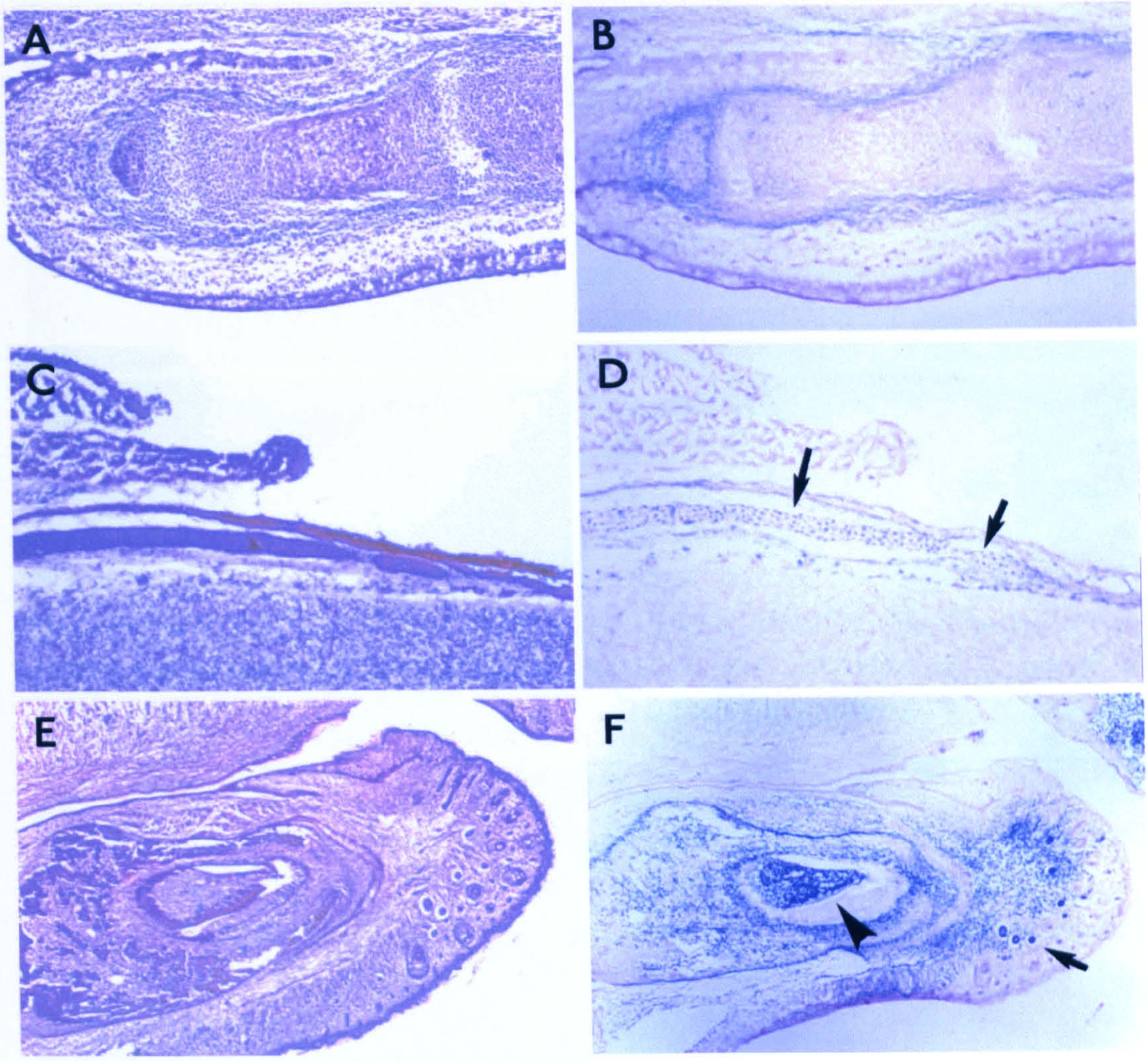


Figure 6.8 – Expression of *Msx1* during late foetal development in *Msx1-lacZ* transgenic mice. Haematoxylin and eosin staining (A, C, E) and β-galactosidase activity (B, D, F) in adjacent longitudinal cryostat sections of embryonic stage 16 (E16) limb digit (A, B), E18 calvaria (C, D) and E18 mandible (E, F) of *Msx1-lacZ* transgenic mice. *LacZ* expression was especially noticeable in the distal tip of the developing digit (B), occipital cartilage of calvaria (arrows, D), tooth germs (arrowhead; F), hair follicles (arrow; F) and the surrounding ossifying regions of the mandible (F) and maxilla (not shown). Magnification x100 A-D and x40 E, F.

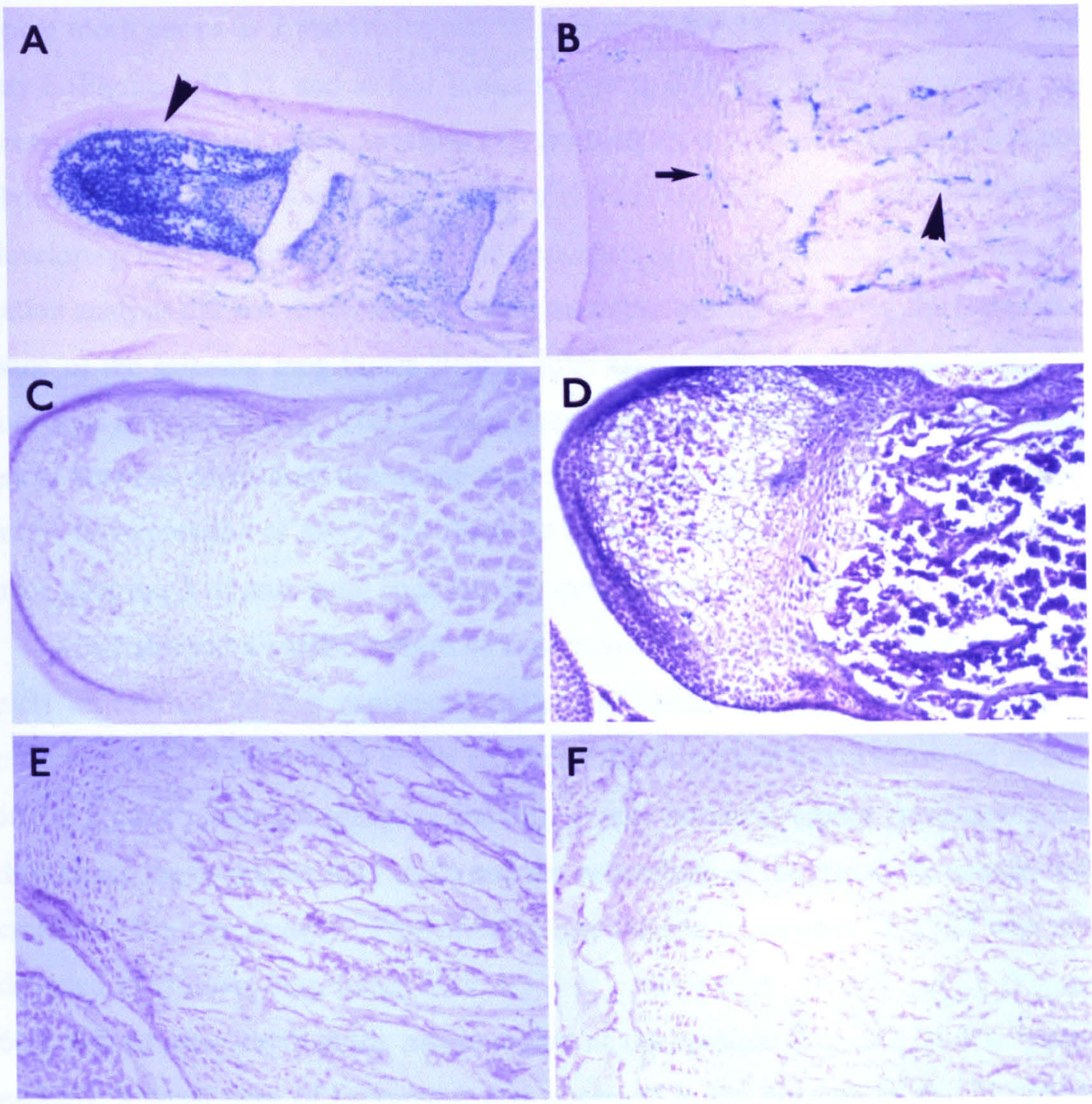


Figure 6.9 – Expression of *Msx1* during post-natal development in *Msx1-lacZ* transgenic mice. β-galactosidase activity (A, B, C, E, F) in longitudinal cryostat sections of post-natal day 1 limb digit (A), day 1 humerus (B), day 8 humerus (C), and day 22 and day 28 femur (E and F, respectively) of *Msx1-lacZ* transgenic mice. *lacZ* expression was especially noticeable in the distal tip of the developing digit (arrowhead in A), and the osteoblast lining the bony trabeculae (arrowhead; B) and developing growth plate (arrow; B). There was an absence of *lacZ* expression in the osteoblasts of long bone tissue following postnatal day 1 (C-F). An adjacent section of the day 8 humerus was stained with haematoxylin and eosin (D) to aid identification of bone structure. Magnification x100.

As was observed above, *Msx1* expression was observed at high levels in developing tooth germs in foetal tissue, and at post-natal day 3 (Figure 6.10 A, B), and day 6 (Figure 6.10 D), and in hair follicles (Figure 6.10 E). *Msx1* was weakly expressed in osteoblasts of the mandible (Figure 6.10 F), maxilla and cranium (data not shown) as late as day 8 of development, and perichondrial regions surrounding the developing digits (data not shown). At post-natal day 1, radioactive *in situ* hybridisation analysis did not show *Msx1* expression in the osteoblasts lining the trabecular bone of the growing long bones (Figure 6.10 G) as observed in similar trabecular locations in Figure 6.9 B. This minor discrepancy may be as a result of strain differences between *Msx1-lacZ* transgenic mice and non-transgenic CD1 mice. Furthermore, no expression of *Msx1* was observed in the osteoblasts of long bones of these mice after day 3 of post-natal bone development (Figure 6.10 H-J).

Msx2 showed a similar pattern of expression to that of *Msx1*. Figure 6.11 depicts an example of this expression pattern for *Msx2* with noticeable expression observed in developing tooth germs and hair follicles both in foetal tissue and as late as day 8 of development (data not shown), in perichondrial regions surrounding the developing digits (Figure 6.11 A-C) and the osteoblasts of the mandible, maxilla and cranium (data not shown). Furthermore, osteoblastic expression of *Msx2* was not detected in growing long bones after day 3 of development up until day 21 (Figure 6.11 F-I) or after day 3 in the suture region of the cranium (Figure 6.11 E). Interestingly, a small core of expression was observed in the cranium at day 21 which was not observed any earlier in development (figure 6.11 J).

Hybridisation of sense riboprobes showed no positive signal of *Msx1* and *Msx2* in all cases, and have only been depicted once for simplicity (Figure 6.10 C, 6.11 D).

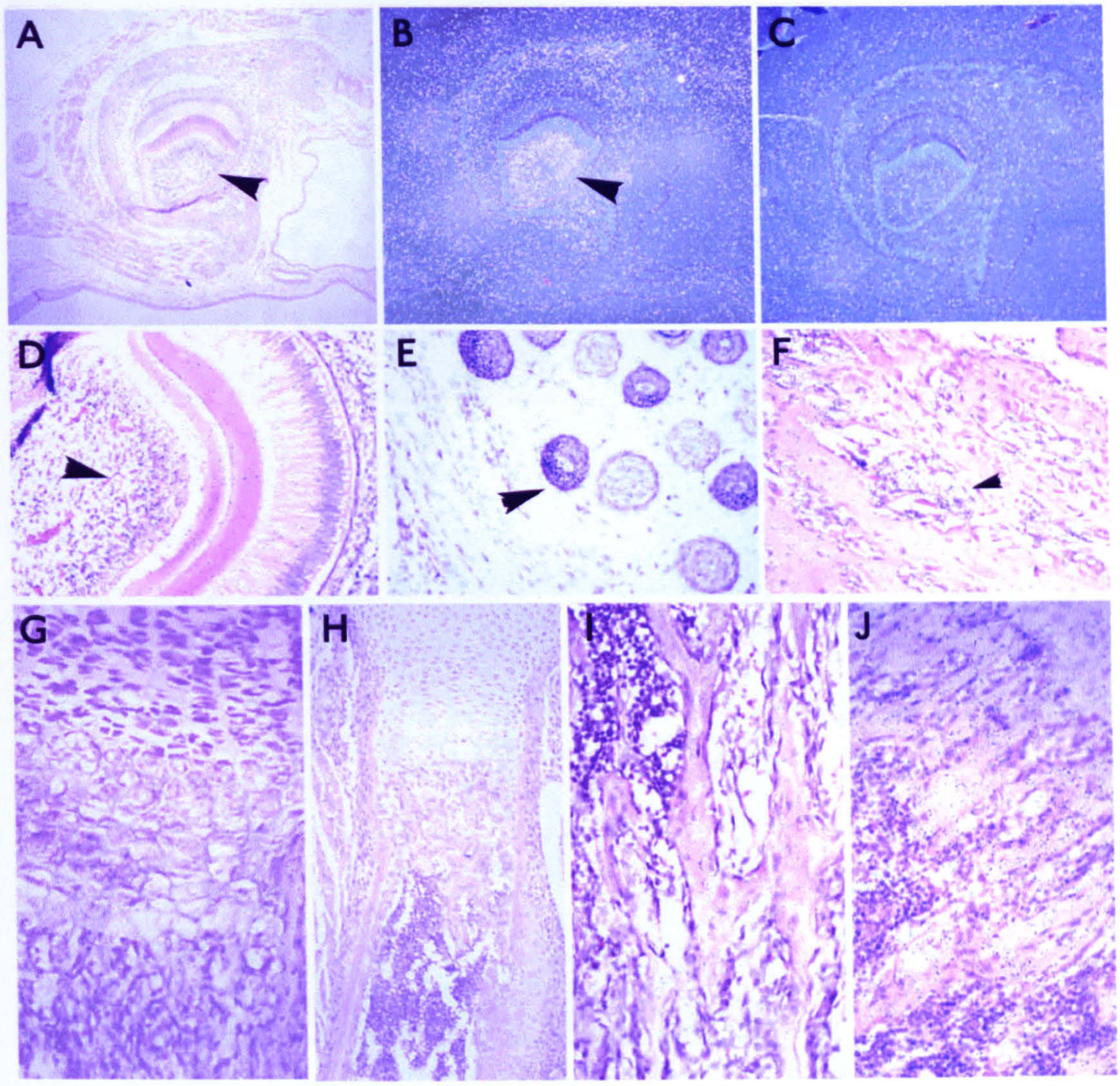


Figure 6.10 – *Msx1* gene expression during post-natal bone development. Radioactive anti-sense (A, B, D to I) and sense (C) *Msx1* riboprobes were used to perform *in situ* hybridisation analysis on longitudinal bone sections from hemi-sected heads and long bones of mice representative of various stages of bone development. Following hybridisation, sections were counterstained with haematoxylin and eosin and mounted for photography. Early post-natal day 3 (A-C), day 6 (D, E), and day 8 (F) show positive expression of *Msx1* in tooth germs (arrowheads in A,B,D), hair follicles (arrowhead, E) and craniofacial bone (mandible; arrowhead in F). However, expression was absent in the long bones from mice at day 1 (G), day 3 (H), day 10 (I) and day 21 (J) of postnatal development. Hybridisation of sense probes gave negative results in all cases (only depicted in C for clarity).

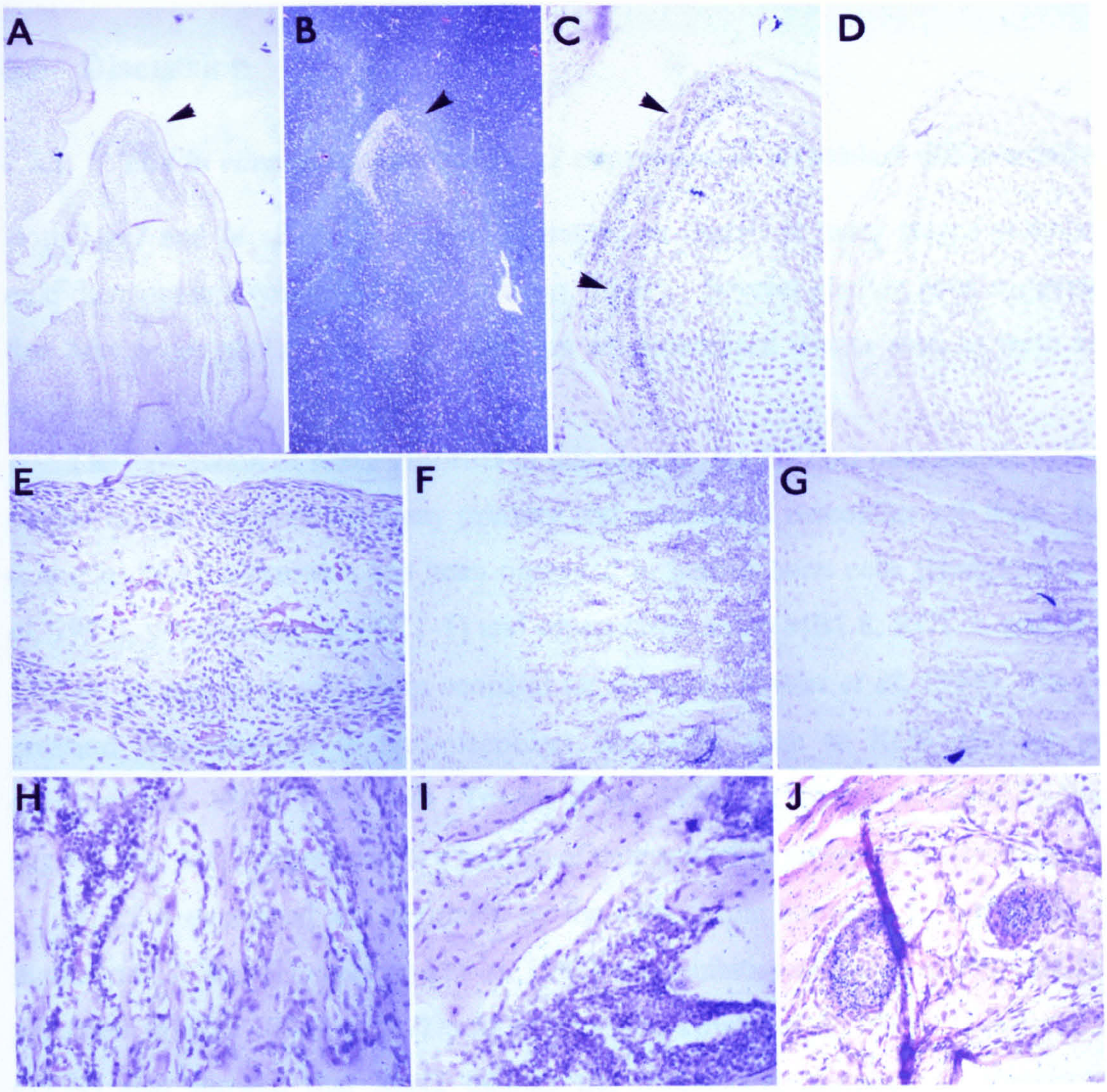


Figure 6.11 – *Msx2* gene expression during post-natal bone development. Radioactive anti-sense (A-C, E-J) and sense (D) *Msx2* riboprobes were used to perform *in situ* hybridisation analysis on longitudinal bone sections from long bones and hemi-sected heads of mice representative of various stages of bone development. Following hybridisation, sections were counterstained with haematoxylin and eosin and mounted for photography. Embryonic expression at E17 shows strong *Msx2* expression in the distal perichondral regions of the developing digit (arrowheads, A-C). However, expression was diminished by post-natal day 3, in the suture (E) and was absent in the growing regions of the long bone (F). Furthermore, *Msx2* expression could not be detected in the limbs of day 6 (G), day 10 (H), or day 21 (I) mice. However, there was a small area of *Msx2* expression in chondrogenic foci in cranial bone (J) of the day 21 mouse. Hybridisation of sense probes gave negative results in all cases (only depicted in D for clarity).

6.6 Discussion

6.6.1 The *in vitro* assessment of *Msx2* expression in osteoblast differentiation

Both *Msx1* and *Msx2* are expressed in numerous tissues at many stages of embryonic development (reviewed by Davidson, 1995). Several studies have suggested that *Msx* genes play a role in development of mineralised tissues such as teeth and skeleton.

The expression of *Msx2* reported in this chapter for SaOS-2 osteoblastic cells is similar to that reported for many primary and established osteoblast cell lines. For example, *Msx2* expression has been observed in human bone cells (Hodgkinson *et al*, 1993), pre-osteoblasts (RCT-1) and osteoblastic cells (MB1.8, RCT 3, and ROS 17/2.8), as well as in cells from neonatal rat calvaria (Towler *et al*, 1994). No expression was detected in non-osteoblast like cells, such as ROS 25/1 or undifferentiated MC3T3-E1 cells (Towler *et al*, 1994). Collectively these findings suggest that *Msx2* expression is a feature of determined or committed osteoblasts.

Msx2 is expressed and regulated by the osteotropic hormone 1,25-$(OH)_2D_3$ in human osteoblasts (Hodgkinson *et al*, 1993) and inhibits expression of the *COL1A1* promoter in ROS 17/2.8 cells (Dodig *et al*, 1996; Towler *et al*, 1994). Towler *et al* (1994) also showed that Msx2 inhibits the osteocalcin promoter in transfected MC3T3-E1 cells, but stimulates the same promoter in ROS 17/2.8 cells. Others have shown that Msx2 represses transcription of osteocalcin in neural crest-derived craniofacial odontoblasts and osteoblasts but not in the mesodermally derived osteoblasts of long bones (Newberry *et al*, 1997; Towler *et al*, 1994; Liu *et al*, 1999; Dodig *et al*, 1996). Osteocalcin and *Msx2* show a reciprocal pattern of expression during tooth development (Bidder *et al*, 1998). The binding of MINT, an Msx2 binding protein, is thought to mediate both the repressor or stimulatory actions of Msx2 by organising the transcriptional complexes on the osteocalcin promoter during craniofacial development (Newberry *et al*, 1999). Another Msx2 binding protein Miz1 (also known as ARIP3) has also been identified but its expression in craniofacial osteoblasts and odontoblasts has not been reported (Wu *et al*, 1997). Further control of Msx2 repressor function at the transcriptional level, is shown with Dlx2, another homeodomain transcription factor expressed in calvarial osteoblasts, which upregulates osteocalcin expression in osteoblasts by antagonising Msx2-

transcriptional repression (Zhang *et al*, 1997; Newberry *et al*, 1998). In addition, Msx1, but not Msx2, may down regulate Cbfa1 expression in the odontoblastic cell line, MO6-G3 (Bin-Wakkach *et al*, 2001).

Gain of function studies in transgenic mice and a dominant mutation in the human gene for *MSX2* result in a craniosynostotic condition in which the cranial suture closes early (Liu *et al*, 1995; Jabs *et al*, 1993; Ma *et al*, 1996; Winograd *et al*, 1997). In contrast, studies in *Msx2* deficient mice show defective proliferation of osteoprogenitor cells at the osteogenic front during calvarial development, a condition found to resemble that associated with human *MSX2* haploinsufficiency in parietal foramina (Satokata *et al*, 2000). *In vitro* studies have confirmed that *Msx2* expression decreases as calvarial osteoblasts differentiate, suggesting that Msx2 prevents differentiation and increases proliferation of osteoblasts at the osteogenic front (Kim *et al*, 1998; Dodig *et al*, 1999; Liu *et al*, 1999). Thus, an understanding of the control of *Msx2* expression by osteotropic factors may be useful in defining some of the molecular mechanisms involved in bone cell differentiation and may help to identify useful therapeutic targets for the treatment of bone diseases such as osteoporosis.

PTH is a well studied regulator of osteoblastic cells *in vitro* and *in vivo*. Its actions can be either stimulatory or inhibitory depending a number of parameters. For example, *in vivo* the anabolic effect of PTH is markedly accentuated when the hormone is administered once daily, such that synthetic PTH fragments are now used for the treatment of osteoporosis (reviewed by Juppner, 1999). In post-menopausal osteoporosis daily subcutaneous injections of PTH is associated with dramatic increases in cancellous bone mass (Lane *et al*, 1998), and a marked reduction in the incidence of new bone fractures (e.g. Reeve *et al*, 1980; Hock *et al*, 1988; Kimmel *et al*, 1993; Hock and Gera, 1992), whereas continuous exposure causes an increase in osteoclast cell number and activity via affects on osteoblasts (Tam *et al*, 1982; McSheehy and Chambers, 1986). PTH can stimulate human osteoblast proliferation *in vitro* (MacDonald *et al*, 1980), possibly via E2F-dependent activation of cdc2 (Onishi *et al*, 1997). PTH can either stimulate or repress osteoblast differentiation and bone-like nodule formation depending on the cell type under investigation (Yee *et al*, 1986; Bellows *et al*, 1990). Additionally, in some cases PTH inhibits collagen synthesis and decreases alkaline phosphatase activity (Kream *et al*, 1886; Majeska

and Rodan, 1982) whereas others the reverse has been found (Martin *et al*, 1985; Hakeda *et al*, 1985). Collectively, these findings suggest that the effects of PTH on cells of the osteoblast lineage are dependant on their state of maturation (Bellows *et al*, 1990; Kim *et al*, 1998; Dodig *et al*, 1999; Liu *et al*, 1999). PTH also stimulated connexin-43 levels in proliferating and maturing osteoblasts but not in differentiated or non-dividing cells, in addition to reducing nodule formation, osteocalcin production, and calcium accumulation (Schiller *et al*, 1997).

In this investigation, PTH displayed no regulatory control of either of the 1.2kb or 2kb *Msx2*-CAT reporter constructs or that of the endogenous gene in the osteoblastic cell lines studied. The action of PTH is dependent on it binding to PTH/PTHrP receptors (PTHR1) and the subsequent stimulation of the adenylate cyclase/cAMP/protein kinase A pathway and, to a lesser extent calcium/inositol triphosphate/PKC pathway. Some of the osteoblastic genes which are regulated via cAMP/protein kinase A pathway include c-*fos*, c-*jun*, alkaline phosphatase, *COL1A1*, *Fgf*-2 (partly mediated by AP-1 proteins), collagenase (in conjunction with Cbfa1), IL-6, LIF, IGF-I, PGHS-2, TGFβ-2, bone sialoprotein, inhibitory cAMP response proteins (ICERs), vitamin D receptor (Pearman *et al*, 1996; Martin *et al*, 1985; Hakeda *et al*, 1985; Hurley *et al*, 1999; Selvamurugan *et al*, 2000; Tetradis *et al*, 1996; Wu and Kumar, 2000; Yang and Gerstenfeld, 1996; Tetradis *et al*, 1998).

Many PTH responsive genes are thought to be secondary targets for hormone action due to the delayed nature of their response and the requirement for ongoing protein synthesis. An example of a primary response gene that PTH transcriptionally regulates is c-*fos*. This is mediated via increased intracellular cAMP signalling which increases phosphorylation of cAMP response element (CRE) and binding of CRE binding protein (CBP) to consensus sequence 5' of the c-*fos* gene (Lee *et al*, 1994; Onyia *et al*, 1995). Analysis of the sequence data for the 1.2kb construct shows that there are no cAMP response element binding sites in this 5' upstream sequence. Gonzalez *et al* (1998) also reported a lack of CBP sites in 4.9kb 5' flanking region of the mouse *Msx1* gene although there were important sites such as AP-1, AP-2, SP1, and RAR present. In the 1.2kb *Msx2* sequence, there are also various AP-1, AP-2, E-box and TDFII binding sites present, which have the potential to be regulated by early response genes, such as c-*fos*. Unfortunately, the se-

quence data for Maxson's 2kb 5' sequence used in the *Msx2*-CAT construct is as yet unpublished (see below). If the work in this chapter had shown some regulation of endogenous *Msx2* by PTH then one could suppose that the 5'regulatory sequences used in the two *Msx2*-CAT constructs were of insufficient length to direct the transcriptional control by PTH signalling. Future work could involve using a 5.2kb 5' flanking sequence of *Msx2* which, when fused to a LacZ reporter to generate transgenic mice, was shown to recapitulate the expression of the endogenous gene during embryonic development in craniofacial tissue, limb mesenchyme, and apical ectodermal ridge (AER), and in early post-natal calvarial suture closure, but was not expressed in the tooth (Liu *et al*, 1994; Liu *et al*, 1999). Transgenic mice with fragments of this 5'sequence deleted showed that the –0.44 and -0.3kb 5' sequences could only drive ectopic expression in AER (Liu *et al*, 1994).

Harris *et al* (1996) also showed a lack of PTH-induced regulation of endogenous *Msx2* using SV40 immortalised human fetal osteoblast cells. Similarly, 1,25-$(OH)_2D_3$, dexamethasone, retinoic acid, and prostaglandins had little effect on *Msx2* expression, whereas BMP2 and TGFβ-1 produced a modest 2-fold increase (Harris *et al*, 1996). This suggests that *Msx2* is unaffected by regulation with osteotropic factors. More recently, Adams *et al* (1999) identified a novel paired class homeobox protein induced by PTH following differential display, suggesting that PTH can regulate the expression of homeobox proteins.

To confirm that the reporter constructs were functional, in the cell lines studied in this chapter, transfected cells were also treated with other factors previously shown to regulate *Msx2* expression. In the preliminary experiments in this chapter, BMP-2, BMP-4 and FGF-8 showed stimulatory activity of the *Msx2* reporter constructs. BMP are thought to be involved in regulating the balance between undifferentiated and differentiated states of osteogenic cells at the osteogenic front and *Msx* genes are involved in this signalling pathway (Kim *et al*, 1998). Furthermore, FGF may act later on in committed osteoblasts. Similarly FGFs have a significant role in bone development highlighted by the dominantly inherited skeletal disorders such as craniosynostosis (Shiang *et al*, 1994, Cohen *et al*, 1995) and FGF receptor (FGFR) mutations in Crouzon syndrome (Fragale *et al*, 1999). Also, signalling through FGFRs can upregulate the osteocalcin promoter (Newberry *et al*, 1996). BMPs and FGFs have been shown to be co-expressed with *Msx* genes at various

sites in the developing embryo, neonatal tooth and suture development, and many studies have suggested that *Msx* genes are downstream targets for these growth factors depending on the factor and tissue studied (Vanio *et al*, 1993; Watanabe and Ide, 1993; Fallon *et al*, 1994; Wang and Sassoon, 1995; Kostakopoulou *et al*, 1996; Kettunen and Thesleff, 1998; Bei and Maas, 1998; Niswander and Martin, 1992; Kim *et al*, 1998; Tucker *et al*, 1998; Graham *et al*, 1994; Shimeld *et al*, 1996; Watanabe and LeDouarin, 1996; Barlow and Francis-West, 1997; reviewed in Davidson, 1995).

The regulatory Smad proteins are downstream mediators of BMP receptor signalling in the cell, and are related to the *Drosophila mothers against dpp (mad)*. Activation of BMP receptors leads to phosphorylation of Smad1 and Smad5 by the type I receptor (previously transphosphorylated by its type II heteromeric partner) followed by their interaction with Smad4. These complexes are translocated to the nucleus where Smad4 functions as a transcriptional coactivator (Derynk *et al*, 1998; Yamaguchi *et al*, 2000). In particular, Smad4 has been demonstrated to be important in the BMP mediated control of *Msx2* expression in the differentiation of ES cells into embryoid bodies (Sirard *et al*, 2000). Most importantly, this work also involved studying the regulation of the larger 1.7kb upstream sequence of the mouse *Msx2* gene in a *Msx2-Luc* construct transfected into fibroblasts. This sequence was shown to be the most responsive to BMP induction via Smad4 binding (R. Maxson's unpublished data). In the 1.2kb *Msx2* construct studied in this chapter, there were four consensus sequences [AGAC] for Smad binding elements as reported by Zawel *et al* (1998). These sequence may be important for the regulation of the 1.2kb construct. Interestingly, Dr. R. Maxson has found inhibitory elements in the 2kb *Msx*-CAT construct which were shown to attenuate the BMP responsiveness (*pers. commun;* unpublished data). BMP proteins have also been shown to complex with A FAST1 transcription factors and interact with DNA at a 6bp and 7bp ARE repeat, however no potential repeat sites were identified in the 1.2kb *Msx2* sequence.

In this study, a preliminary experiment also showed that the *Msx*-CAT constructs responded to stimulation by Sonic hedgehog (Shh), although this may be an indirect effect, mediated by the induction of BMP expression. Shh and Indian hedgehog (Ihh) are involved in skeletal formation during development and during skeletal repair (reviewed by Yamaguchi *et al*, 2000). The regulation of osteoblast

differentiation is thought to involve the up-regulation of BMPs. Shh and Ihh expression have been previously shown to correlate with BMP signalling at various sites in the mouse embryo and are reported to regulate BMP expression in various mesenchymal sites and osteoblastic cells lines (Bitgood and McMahon, 1995; Yamaguchi *et al*, 2000). Indeed ectopic expression of Shh leads to bone formation. Recent studies have shown that Shh stimulates BMP expression through the Shh transcriptional mediators Glil or Gli3 (Kawai and Sugiura, 2001). In addition, the Shh protein may interact with BMPs and lead to control of *Msx* expression at the osteogenic front through a patched-dependent pathway, which may be involved in the prevention of early sutural closure (Kim *et al*, 1998). In this work, expression patterns for *Msx* and BMPs were found to be discontinuous along the osteogenic fronts reflecting a patched pattern and thus resembling *Shh* and *Ptc* expression. In addition, studies in *Drosophila* have shown that the *msh* gene is also influenced by hedgehog signalling (D'Alessio and Frasch, 1996). Finally, in the mouse tooth germ, Msx1 has been demonstrated to be required for the induction of Patched (*Ptc*) by Shh (Zhang *et al*, 1999). Ptc is the Shh transmembrane receptor, that functions as a negative regulator in the Shh signalling pathway. To further investigate the mechanism(s) by which Shh regulates *Msx* expression it will be necessary to monitor the levels of BMPs and Gli proteins following Shh stimulation. Alternatively, cells could be treated with Shh in the presence of BMP antagonists, such as Noggin proteins.

The small effect of 1,25-$(OH)_2D_3$ was not significant in this chapter since there was a great deal of variability between experiments. From research previously carried out in our laboratory, no vitamin D response elements have been identified in the 1.2kb 5' regulatory sequence and this construct was not found to be regulated by 1,25-$(OH)_2D_3$ in the human MG-63 osteoblast cell line and ROS 17/2.8 cells (Hodgkinson, 2000; unpublished data from PhD thesis). In addition, Dr. R Maxson's group have not reported any VDRE the 5.2kb regulatory sequence (Liu *et al*, 1994). However, using RT-PCR techniques, Hodgkinson was able to show that 1,25-$(OH)_2D_3$ could regulate *Msx2* expression in primary cultures of normal human bone cells (Hodgkinson *et al*, 1993). This stimulation may have been indirect through the signalling of other osteotropic pathways. The results of experiments in which cells were exposed to 1,25-$(OH)_2D_3$ in the presence of cyclohexamide, an

inhibitor of protein synthesis, are consitsent with this possibility (Brown, J. and Beresford, J.N., personal communication).

6.6.2 Investigation into the expression of *Msx* genes in adult murine osteoblasts

The aims of this project were to investigate further the expression of *Msx1* and *Msx2* genes during bone development and osteoblast differentiation by studying their expression in post-natal bone. Using both LacZ reporter gene expression analysis and *in situ* hybridisation the postnatal expression of both *Msx1* and *Msx2* could not be demonstrated.

As mentioned above, before the work on this chapter was started, most of the research implicating *Msx* genes in the control of osteoblast differentiation and tissue mineralisation in osseous tissues had suggested a role for these genes in cells derived from the neural crest but not in the mesodermally derived osteoblasts of long bones. This is in contrast to Cbfa1 which provides a global control of the osteoblast phenotype of the entire skeleton, including the skull (Ducy *et al*, 1997, Komori *et al*, 1997, Otto *et al*, 1997).

In this investigation using bones from wild-type mice and *Msx1-lacZ* knock-in mice, there was no observable post-natal bone expression of either *Msx1* or *Msx2*. In contrast, expression was detected in the calvaria, tooth and long bones of embryonic or early post-natal mice (less than 3 days of age), as has been reported previously (Hill *et al*, 1989; Robert *et al*, 1989; Davidson *et al*, 1991; Monaghan *et al*, 1991; MacKenzie *et al*, 1992; Reginelli *et al*, 1995; Kim *et al*, 1998; Liu *et al*, 1999). A minor discrepancy is that in Liu *et al* (1999), using a *Msx2-lacZ* knock-in mouse model, found that *Msx2* continued to be expressed in the cranial sutures as late as P4. However, promoter activity was reduced by this stage and exhibited a punctuate pattern in undifferentiated osteoblasts in the outer margin of the osteogenic fronts (a pattern which was similar to that found by Kim *et al*, 1998). Had the work described here looked at more serial sections of the calvaria, expression may have been observed at later time points.

The results in this investigation are consistent with those of Nowroozi *et al* (1996), who found that in adult *Msx2-lacZ* knock-in transgenic mice, *Msx2* expression was high in pre-osteoblasts and osteoblasts in calvarial sutures but not in neighbouring periosteal regions. *Msx2* expression was detected in the mandible and max-

illa, but not in the periosteal areas of long bones, although some chance staining was observed in individual cells neighbouring the metaphysical cartilage (Nowroozi *et al*, 1996).

Recently, there has been a report that there is strong expression of *Msx2* in the long bones of mice as late as post-natal day 30 (Satokata *et al*, 2000). However, these mice had defective proliferation of osteoprogenitors at the osteogenic fronts during calvarial development (Satokata *et al*, 2000). In addition, they had defects in endochondral bone formation and in the axial and appendicular skeleton, there were post-natal defects in the PTH/PTHrP receptor signalling and in the expression of osteoblast differentiation marker genes (Cbfa1, alkaline phosphatase, bone sialoprotein, and osteocalcin). This work suggests that Msx2 is required for both chondrogenesis and osteogenesis. Of particular relevance to the work presented here, these workers also found some *Msx2* expression in proliferating chondrocytes and in the osteoblasts of the periosteum of wild type mice at post-natal age 30 (Satokata *et al*, 2000).

Since the completion of this study, Orestes-Cardoso *et al* (2001) have reported adult expression of *Msx1* using the same strain of n*lacZ* knock-in transgenic mice. Their work suggests a role for *Msx1* in bone growth in the osteogenic fronts of sutures, growth plate cartilage, and periosteum, as well as skeletal patterning of distal extensions of the mandible, limbs, sutures, and alveolar bone associated with the teeth. Furthermore, *Msx1* expression was found to be localised to chondrocytes and osteoclasts, in addition to osteoblasts. When the work described here was initiated, bone samples were fixed and decalcified using standard histological procedures of formalin fixation and EDTA decalcification (see section 2.5.6 for details). However, this procedure leads to an apparently high background level of X-gal staining in both transgene positive and transgene-negative samples. In the paper by Orestes-Cardoso *et al* (2001), the workers discuss the need to fix for a minimum of 30 minutes and titrate the quantities of X-gal stain to ensure specific staining of positive tissue. This is something which was not investigated here and for this reason alone, it is difficult to interpret the discrepancies between the findings of the two studies. It should be noted, however, that Orestes-Cardoso *et al* did not substantiate their work with transgene negative samples or *in situ* hybridisation analysis.

6.7 Conclusions

PTH showed no regulation of either endogenous or exogenous *Msx2* expression. However, BMPs were shown to stimulate the *Msx2*-CAT constructs. Using *Msx1-lacZ* transgenic mice, LacZ expression was only detected in mineralising tissues of the foetus and neonate, with no LacZ expression observed after birth. Similar results were found when endogenous Msx1 and Msx2 expression patterns were studied.

7. General Discussion

The work described in this thesis has helped to elucidate further the roles of c-Fos and Msx transcription factors in bone cell differentiation and in health and disease. The role of c-Fos in osteoclast biology was investigated using an *in vivo* transgenic approach, and its potential function in regulating osteoblast differentiation and apoptosis was assessed using a tightly controlled gene induction system *in vitro*. Finally, the regulation of Msx gene expression in osteoblasts was investigated in response to osteotropic hormones.

That c-Fos plays a major role in bone biology came from earlier studies using c-*fos* knockout mice in which it was found that c-Fos was essential for osteoclast development (Grigoriadis *et al*, 1994), as well as from studies demonstrating that c-*fos* is highly expressed in lesions from patients with Paget's disease. In this thesis, the *in vivo* effects of c-*fos* over-expression in osteoclasts was addressed using TRAP-c-*fos*LTR transgenic mice. Several founder mice developed bone lesions and tumours which contained a large numbers of multinucleated cells that expressed several osteoclast-specific markers and were functional as evidenced by the presence of highly remodelled bone. However, the reasons for the apparent increase in the number of osteoclasts is not yet clear: One possibility could be because of increased osteoclast differentiation from circulating precursors, as a result of a positive feedback signalling from increased bone remodelling and a disruption of the balance between bone formation and resorption. Alternatively, it could be due to increased osteoclast survival as evidenced by the high levels of Bcl-2 expression in the transgenic osteoclasts. c-*fos* has been demonstrated to be downstream of RANK/RANKL in the signalling pathway mediating osteoclast differentiation (Matsuo *et al*, 2000). Future studies should therefore investigate whether high c-Fos expression has had a positive feedback effect in modulating expression of these key osteoclast differentiation factors, in addition to changes in expression of *Opg*, which may help to ascertain any imbalance in bone formation versus bone resorption.

An additional line of future experimentation should also include a comparison of the effects of the TRAP-c-*fos*LTR construct with one in which the long terminal repeat (LTR) has been removed. As described in section 1.6.4, this LTR fragment is required for stable expression of the c-*fos* transgene *in vivo*, and is necessary for transformation of osteoblasts, as exemplified in H2-c-*fos*LTR mice (Grigoriadis *et al*, 1993). Although the TRAP promoter has been a useful system for targeting gene

expression to osteoclasts (e.g., Roodman *et al*, 1996), and we have achieved high levels of exogenous c-Fos protein in osteoclasts, we cannot completely rule out the possibility that the LTR fragment may be overriding the TRAP promoter specificity, leading to additional inappropriate expression in osteoblasts, and generation of a tumour phenotype. This can be addressed by generating additional mice using TRAP-c-*fos* constructs which lack the LTR but contain another suitable polyA signal. Alternatively, as more promoter sequences become characterised and more information on osteoclast markers becomes available, an alternative promoter could be used for the targeting of c-*fos* to osteoclast at different stages of the differentiation sequence. For example, the RANK or MMP-9 promoters could be used to target cells at early stages of osteoclast development, whereas the promoters for vacuolar-ATPase, cathepsin K or carbonic anhydrase II could be used for targeting to more mature osteoclasts. Enforced *in vitro* expression of c-*fos* in osteoclasts could be investigated using the RAW264.7 macrophage cell line stably transfected with the inducible c-Fos construct utilised for the MC3T3-E1 cell line (chapters 4 and 5; see also Thomas *et al*, 2000).

Clearly, further experiments are required to confirm unequivocally the appropriate targeting of c-Fos in osteoclasts and assess the consequences for their functional activity. To achieve this more transgenic founder mice need to be generated which can be bred to give transgenic families, thereby allowing more *in vivo* and *in vitro* studies to be performed. In addition to studies mentioned above, *in vitro* studies should, for example, investigate the osteoclastogenic capacity and function or altered gene expression of haematopoietic spleen precursors, by culturing cells derived from tumours in the presence of RANKL.

This thesis also identified an *in vitro* role for c-Fos in the proliferation, differentiation and apoptosis of osteoblasts *in vitro*, despite the fact that c-Fos has been shown to be dispensable for these processes *in vivo* (Field *et al*, 1992; Brusselbach *et al*, 1995; Johnson *et al*, 1992; Wang *et al*, 1992; Gajate *et al*, 1996, Roffler-Tarlov *et al*, 1996). Using *in vitro* systems, the overexpression of c-Fos in osteoblasts may have resulted in the attenuation of the osteoblast phenotype, possibly through an increase in proliferation and decrease in maturation. Previous studies have shown that AP-1 proteins follow a sequential pattern of gene expression during osteoblast differentiation (McCabe *et al*, 1995; Banerjee *et al*, 1996). The work

presented here has shown that this balance, in particular the regulation of BMP-2 induced alkaline phosphatase activity, can be disrupted by the over-expression of c-*fos*. The inhibition of alkaline phosphatase activity may occur as a direct result of transcriptional control of alkaline phosphatase expression via AP-1 binding sites in regulatory sequences (Owen *et al*, 1990; Slack *et al*, 1995) or through indirect regulation of BMP downstream signalling molecules including SMAD6 and SMAD7, BMP receptors or other BMP target proteins. These possibilities remain to be investigated.

One interesting line of future investigation would be to induce c-*fos* expression at various points in the differentiation sequence. This is possible only because of the tight control offered by the Tc-regulatable system. This may help to delineate the potential effects of c-Fos on the osteoblast mineralisation process, which may by overridden in the presence of c-Fos inducing a hyperproliferative advantage over the cells. In the normal osteoblast differentiation sequence the proliferation period supports the biosynthesis of a type-I collagen extracellular matrix, which continues to mature and mineralise. This matrix then suppresses osteoblast proliferation (Stein *et al*, 1990). During the process of extracellular matrix maturation and mineralisation, c-Fos is known to stimulate the production of collagenases (Angel *et al*, 1987; Schonthal *et al*, 1988; Gack *et al*, 1994; Winchester *et al*, 2000), which are necessary for the break down of this matrix during bone remodelling. Thus, one could speculate that in the KT1.5 cell system ectopic c-Fos expression is affecting the formation of a competent extracellular matrix via transcriptional repression of the genes for extracellular matrix proteins and for collagenase. Future work should investigate the changes in levels of expression of additional osteoblast differentiation markers, such as collagen, BSP, osteocalcin and MMP-13 in KT1.5 cells following ectopic c-*fos* expression. Furthermore the effect of c-Fos over-expression on the response of cells to osteotropic factors such as 1,25-$(OH)_2D_3$ and PTH needs to be investigated. Recently, Demiralp *et al* (2001) reported that c-Fos may have a role in the PTH induced mineralisation, since osteoblasts from c-*fos* deficient mice showed decreased mineralisation compared to wild type cells. Based on the fact that cultures of KT1.5 cells do not fully mineralise, primary osteoblasts derived from H2-c-*fos*LTR mice could be used for these studies.

The inducible overexpression of c-Fos in osteoblastic cells also resulted in increased apoptosis induced by diverse mechanisms. This is a highly significant finding, and involves a mechanism that is independent of caspase activation but dependent on specific cell cycle regulatory genes. Our laboratory has recently demonstrated that ectopic c-Fos perturbs osteoblast cell cycle progression (Sunters *et al*, 2000), and the work in this thesis provides a novel link between cell cycle control and programmed cell death in osteoblasts. Many studies have demonstrated a link between proliferation and apoptosis following oncogene expression (reviewed Evan and Littlewood, 1998), with c-Myc being a prime example (Prendergast, 1999). However, the results from this thesis suggest that c-Fos induces apoptosis through different mechanisms to that of c-Myc, potentially through the regulation of CDKs. Clearly, future experiments will be designed to elucidate the mechanisms of c-Fos-induced apoptosis as it relates to cell cycle regulation. These should include an analysis of CDK levels and cell cycle kinetics during the apoptosis program. Additionally, cell cycle regulatory factors may also be involved in the mechanisms of c-Fos-induced apoptosis, that may be stimulating CDK activity. For example, increased c-Fos expression may potentiate expression of the E2F transcription factor. Stimulated E2F may proceed to potentiate cell death by blocking anti-apoptotic signalling pathways including the down regulation of TRAF2 and inhibiting the activation of anti-apoptotic signals such as NF-κB. c-Fos may also directly inhibit anti-apoptotic pathways such as NF-κB, and JNK/SAPK activation pathways. Furthermore, downstream targets of CDKs that may result in the progression of apoptosis need to be elucidated to confirm that the resulting apoptosis is either a direct effect of altered cell cycle machinery or indirect regulation of factors important for apoptosis such as Bcl family proteins, nuclease activation, or p53 activation (King and Cidlowski, 1995).

Future work could also investigate the regulation of other CDK inhibitors such as p27 in KT1.5 cells following ectopic c-Fos expression. In the normal environment, p27 acts to constrain CDK2 activity and p27 levels have been shown to be down-regulated in cells undergoing apoptosis (Frost and Sinclair, 2000). Indeed, p27-/- fibroblasts show increased apoptosis (Hiromura *et al*, 1999). In addition, p16 and p57 have been reported to inhibit apoptosis through CDK-dependant mechanisms (Wang and Walsh *et al*, 1996; Yan *et al*, 1997).

Also, other apoptotic signalling pathways should be investigated to further understand the mechanisms of c-Fos-induced apoptosis. Siegmund *et al* (2001) recently reported a role for c-Fos in a FasL- and TRAIL-mediated stimulation of proliferation. Here c-Fos was up-regulated via Fas-associated death domain protein (FADD), FADD-like interleukin-1-converting enzyme (FLICE) inhibitory protein (cFLIP) and caspase-8. However, caspase activity seemed to be dispensable in this process as Z-VAD-fmk had no inhibitory effect. In contrast, Maclaren *et al* (2001) demonstrated recently that v-Jun induced apoptosis via a pathway that is dependant on cytochrome c, but independent of the p53 and FADD/caspase-8 signalling pathways. PTH has been shown to inhibit osteoblast apoptosis induced by dexamethasone and Etoposide via a process that involved the activation of adenylate cyclase, but which could not inhibit apoptosis induced by TNF-α (Jilka *et al*, 1999). It will therefore be important to determine whether PTH can prevent the induction of apoptosis induced by ectopic c-Fos expression.

A role of c-Fos in osteoblast apoptosis has not been shown previously. Research has suggested that c-Fos is not necessary for apoptosis in normal mouse development, based on the results from c-Fos deficient mice. However, the study of apoptosis in osteoblasts from mutant mice has not been reported, and thus it would be useful to investigate this process in primary osteoblast cultures derived from these mice. Work by Ethaar El-Emir (PhD thesis) in our laboratory has suggested that osteoblasts derived from tumours of c-*fos* transgenic mice (H2-c-*fos*LTR transgenic mice; Grigoriadis *et al*, 1993) are resistant to apoptosis, based on *in situ* TUNEL analyses in late-stage tumours, and *in vitro* studies on tumour-derived cell lines. However, her research did not include cells derived from mice early on during oncogenic transformation of osteoblasts. Thus future research should extend to studying apoptosis in osteoblast cells derived from these mice at early stages following c-Fos transgene expression; i.e. before the development of large tumours by which time other genetic changes will have surely taken place. This would also help to confirm the results observed in this thesis with respect to the KT1.5 cells. A comparison of changes in gene expression between non-transformed and transformed cells following apoptosis could be used to elucidate mechanisms of the osteoblast's resistance to apoptosis, including an up regulation of Bcl proteins and potential down regulation of p53 and CDK inhibitors such as p21 and p27.

The c-Fos induced apoptosis observed here may involve caspase-independent pathways. However, other caspase inhibitors and higher concentrations of DEVD-CHO need to be investigated. To further explore the potential importance of the caspase-independent signalling pathways it will be important to investigate the release of factors such as AIF (Susin *et al*, 1999). However, this will also require the analysis of other earlier endpoint/markers of apoptosis such as cytochrome c release, to ensure other pathways have not bypassed the requirement for caspases.

Finally, the work described here has helped to address the regulation of Msx homeobox containing proteins in osteoblasts. One of the original reasons for this research was to identify potential therapeutic targets for the treatment of bone diseases, such as osteoporosis. PTH is currently used for the treatment of osteoporosis, but as described previously, its effects vary according to the mode of administration, and the ideal therapeutic agent needs to increase bone mass, without effecting bone resorption. The results of this study suggest that Msx2 is not a candidate protein, however, this does not eliminate the possibilities that other homeobox genes may exist which can be regulated by PTH. Indeed, a novel paired class homeobox protein was shown recently to be regulated by PTH (Adams *et al*, 1999). Thus, additional PTH-regulated homeobox proteins may be identified in the future, possibly by the use of DNA microarrays.

This study has also assessed the regulation of two *Msx2* reporter gene constructs in MC3T3-E1 cells and SaOS-2 cells, which are representative of cells early and late in the osteoblast differentiation sequence. By using cells at these extremes, however, effects of PTH on *Msx2* expression in cells at intermediate stages of differentiation may have been missed. As described in chapters 4 and 5, the MC3T3-E1 cell line can be used to study cells at different stages of osteoblast differentiation. An ideal experiment, therefore, would be to transiently transfect these cells with the 1.2kb or 2kb *Msx* constructs or, better still, the longer 5.2kb construct of Maxson *et al* at various stages of differentiation. Unfortunately, at the time the studies described in this thesis were conducted the latter construct was not available.

In conclusion, the work described in this thesis has provided several novel insights into the biological roles of c-Fos and Msx transcription factors in the control of osteoclast and osteoblast proliferation, differentiation and survival.

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9. Appendix

9.1 The p*Msx2*-CAT construct

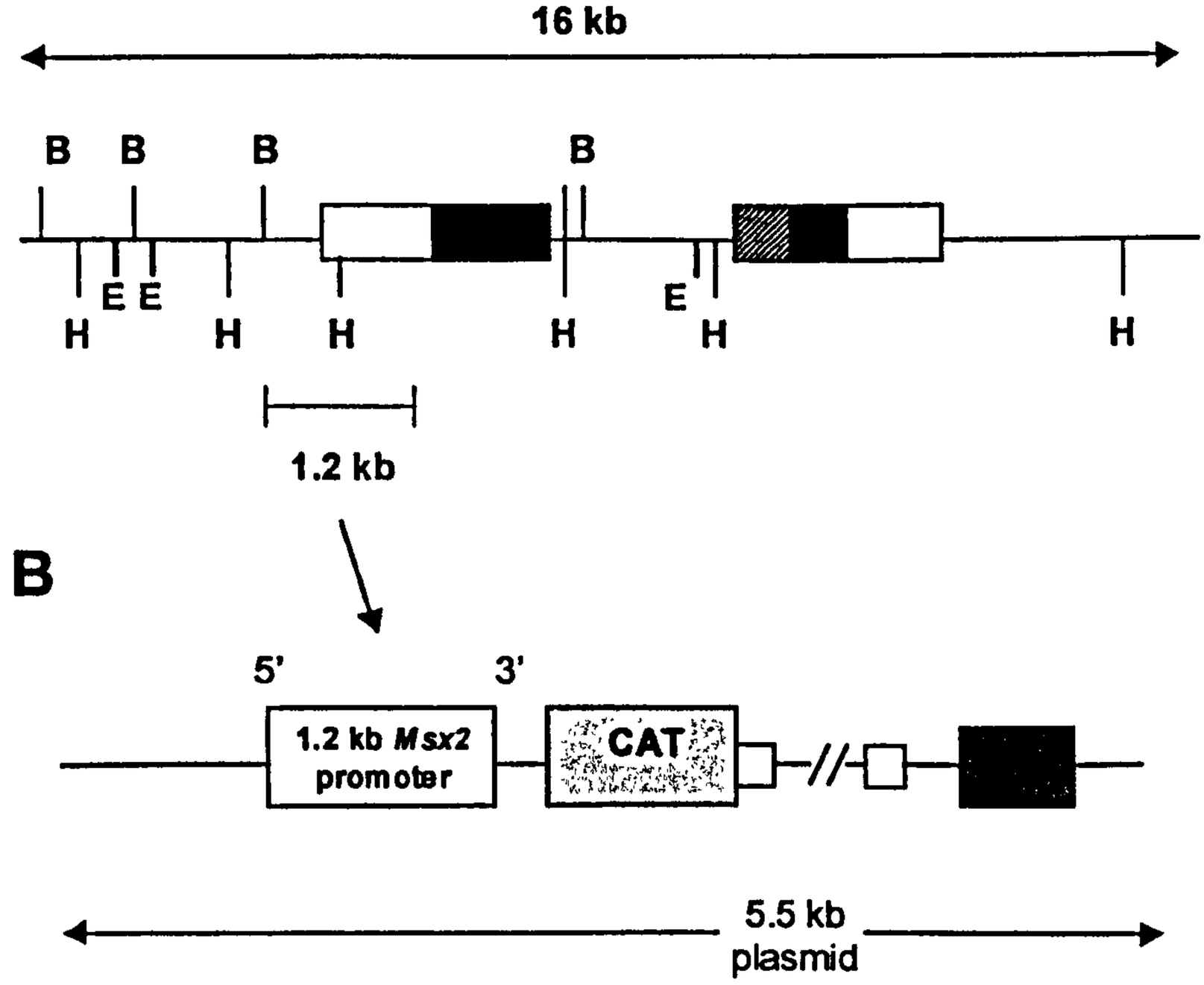

Figure 9.1 – Diagram of the p*Msx2*-CAT reporter construct. (Adapted from Hodgkinson, 2000 PhD thesis). A. Representation of a 16kb genomic clone used to prepare the 1.2kb regulatory sequence (previously isolated by Bell *et al*, 1993), which contained the first and second exons of *Msx2*. The black boxes represent the two coding regions of each exon and hatched region of the homeobox. Open boxes represent transcribed but not translated regions. All HindII (H), BamHI (B) and EcoRI (E) sites are shown. The 1.2kb sequence used in the p*Msx2*-CAT reporter construct is in a region of a BamHI fragment represented by a bar beneath the diagram. This diagram is not to scale. B. Linear representation (and not to scale) of the p*Msx2*-CAT reporter construct. The *Msx2* fragment had been cloned into the Promega pCATbasic vector and is shown upstream of the CAT coding sequence in this plasmid.

JOURNAL OF BONE AND MINERAL RESEARCH
Volume 14, Supplement 2, 1999
Blackwell Science, Inc.

A Putative Role for c-Fos in the Pathophysiology of Paget's Disease

KAREN E. BEEDLES,[1,2] PAUL T. SHARPE,[2] ERWIN F. WAGNER,[3] and AGAMEMNON E. GRIGORIADIS[1,2]

ABSTRACT

The molecular mechanisms underlying Paget's disease and subsequent osteosarcoma formation are not well understood. In this study, we aim to delineate the function of the c-Fos oncogene in Paget's disease using transgenic mice, based on previous findings that c-Fos is highly expressed in Pagetic osteoclasts and that c-Fos is an essential gene for osteoclast differentiation and skeletal neoplasia. We have generated transgenic mice in which c-Fos is overexpressed specifically in osteoclasts using the tartrate-resistant acid phosphatase (TRAP) promoter, and five founder mice have been identified. All transgene-expressing animals developed severe bone remodeling lesions, some of which progressed to large bone tumors. Histopathologic analysis indicated that the lesions contained a marked increase in the number of osteoclasts that contained a large number of nuclei. Osteoclasts were identified by histochemical staining for TRAP and by in situ hybridization for matrix metalloproteinase-9 (MMP-9) expression. Moreover, transgenic osteoclasts, and in some cases, osteoblasts and chondrocytes, expressed high levels of c-Fos protein as judged by immunocytochemistry. This phenotype of increased osteoclast number and activity, together with an apparently high rate of bone turnover, resembles some characteristics of Paget's disease. These data therefore support an important function for c-Fos in the Pagetic phenotype, and further support the notion that this gene is important in osteoclastogenesis and in bone remodeling disorders. (J Bone Miner Res 1999;14, suppl. 2:21–28)

INTRODUCTION

PAGET'S DISEASE OF BONE is a chronic skeletal disorder that affects 4–8% of the elderly population worldwide and is characterized by areas of rapid turnover, deformity, and degeneration.[1] Furthermore, patients with the disease have an increased risk of malignant transformation and are 30 times more likely to develop osteosarcomas.[1] The primary lesion in Paget's disease is thought to involve bone-resorbing osteoclasts, which are increased 50–100-fold both in number and multinuclearity, resulting in an increase in bone turnover. The specific causes of the apparent increase in osteoclast activity, in addition to the molecular mechanisms underlying cellular transformation and oncogenesis, are poorly understood. Despite this, several candidate genes have been associated with Paget's disease. For example, the cytokine interleukin-6 (IL-6) is apparently up-regulated specifically in pagetic osteoclasts, which constitutively express IL-6 receptors and the transcription factor nuclear factor-IL-6 (NF-IL-6), suggesting a possible autocrine role for IL-6.[2,3] There is also increasing evidence supporting a genetic component to Paget's disease, with both Paget's and the Paget's-related disease familial expansile osteolysis (FEO) being mapped to a susceptibility locus on chromosome 18q in some families.[4,5] Moreover, the anti-apoptotic gene Bcl-2 has also been mapped to 18q and recent preliminary data indicate that Bcl-2 expression is up-regulated in Paget's disease.[6] Finally, as described below, earlier studies analyzing the expression of c-Fos have demonstrated that this proto-oncogene is apparently up-regulated in pagetic osteoclasts[7] providing an additional candidate molecule for explaining the possible molecular

[1]Department of Orthodontics and Paediatric Dentistry, GKT Dental Institute, Guy's Hospital, London, United Kingdom.
[2]Department of Craniofacial Development, GKT Dental Institute, Guy's Hospital, London, United Kingdom.
[3]Research Institute of Molecular Pathology (IMP), Vienna, Austria.

basis of Paget's disease, including the potential for cellular transformation and oncogenesis.

We have focused extensively on identifying potential roles for c-Fos in bone development and bone disease using gain-of-function and loss-of-function approaches.[8] c-*fos* is the cellular homolog of v-*fos* that was originally identified in the FBJ and FBR murine sarcoma viruses (MSVs). The c-Fos oncoprotein is a major component of activating protein-1 (AP-1) transcription factor complex, and is part of a multigene family of nuclear proteins, including other c-Fos-related (FosB, Fra-1, Fra-2) and c-Jun-related (c-Jun, JunB, JunD) proteins.[9] The function of c-Fos is dependent upon formation of heterodimers with specific JUN/ATF-2 family members and subsequent binding to AP-1 consensus sequences of regulatory regions of specific genes. As an immediate early gene, c-Fos is associated with a variety of biological processes, ranging from transformation to cell cycle progression and differentiation.[9] While the gene can be induced in a wide range of both embryonic and adult tissues and cell lines, the biological functions of c-Fos have been primarily delineated using transgenic and knock-out mice.[8] Transgenic mice overexpressing c-*fos* using ubiquitous promoters develop remodeling osteosarcomas due to specific c-Fos-dependent oncogenic transformation of osteoblasts.[10] Moreover, histopathologic analysis revealed many features that resemble Paget's disease.[10–12] In contrast, c-Fos knock-out mice lack osteoclasts and develop the bone remodeling disease osteopetrosis,[13,14] clearly demonstrating that c-Fos is an essential gene for osteoclast differentiation and bone remodeling in general.[8] Thus, the gain- and loss-of-function analyses have clearly implicated the c-Fos proto-oncogene and AP-1 transcription factor in both osteoclast differentiation and in bone cell transformation and tumor formation, both processes hallmarks of Paget's disease.

To further examine the role of c-Fos in Paget's disease, and consistent with the observations that osteoclasts in pagetic bone express high levels of c-Fos,[7] we have generated mice in which c-*fos* is overexpressed specifically in osteoclasts. To this end, we have used the promoter for tartrate-resistant acid phosphatase (TRAP), which is expressed at high levels in osteoclast precursors and differentiated osteoclasts, and which has been shown previously to function reliably for targeting transgenes to osteoclasts in mice.[15–17] Our results suggest that when high levels of c-Fos are expressed in osteoclasts, the mice develop a marked bone remodeling phenotype reminiscent of Paget's disease, such as the presence of large numbers of osteoclasts containing many nuclei, and in some cases, the development of large tumors.

MATERIALS AND METHODS

Construction of TRAP-c-fosLTR and generation of transgenic mice

The murine TRAP promoter (pBS-TRAP; obtained from Dr. G.D. Roodman, San Antonio, TX, U.S.A.[15]) was fused to a full-length genomic sequence for the murine c-*fos* gene in which the 3′ mRNA destabilizing sequences have been replaced by a 3′ long-terminal repeat (LTR) from the FBJ-MSV (TRAP-c-*fos*LTR) (Fig. 1).[11] The construct was microinjected (without plasmid sequences) into fertilized eggs according to standard procedures, followed by transfer into foster females. Founder animals were screened by Southern blot analysis of tail DNA for the presence of the transgene, and radiographs were taken on anesthetized mice.

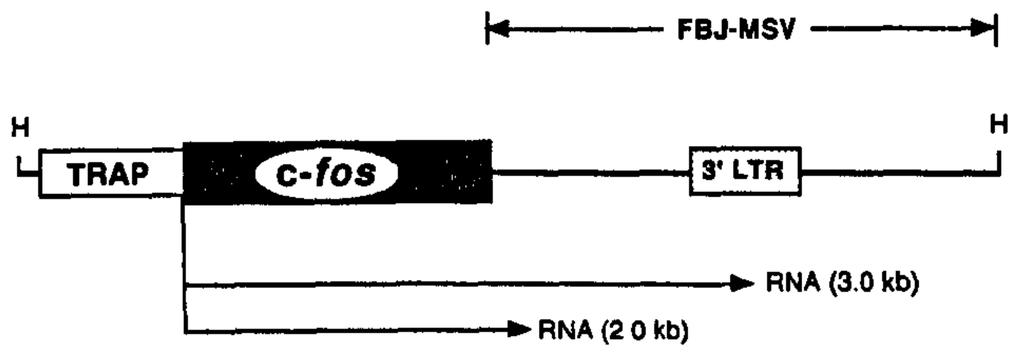

FIG. 1. The DNA construct (TRAP-c-*fos*LTR) used for the generation of TRAP-c-*fos*LTR transgenic mice. The murine TRAP promoter (pBS-TRAP) was fused to a full-length genomic sequence for the murine c-*fos* gene in which the 3′ mRNA destablizing sequences have been replaced by a 3′ LTR from the FBJ-MSV.[11] Two exogenous c-*fos* transcripts are synthesized as indicated. H, *Hind*III.

Northern blot analysis

Northern blot analysis was performed on poly(A)$^+$ RNA isolated from tissue samples of normal and tumor tissues. RNA isolation and hybridization using an 800-bp *Bam*HI fragment from v-*fos* was performed as described previously.[10]

Histopathologic analysis

Transgenic mice were killed by cervical dislocation and tissues were fixed immediately in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 24–48 h and decalcified in either 0.5 M EDTA or 5% formic acid. Material was paraffin embedded following dehydration and vacuum infiltration, 5 μm sections were cut and mounted on 3-aminopropyltriethoxysilane (TESPA)-coated slides and stained by hematoxylin and eosin. For identification of osteoclasts, sections were stained for TRAP in the presence of 50 mM sodium tartrate as described previously.[14]

In situ hybridization of bone from TRAP-c-fosLTR mice

Sense and antisense matrix metalloproteinase-9 (MMP-9) riboprobes were synthesized using a murine MMP-9 cDNA probe (323 bp *Sma*I-*Eco*RI fragment[14,18]) and labeled with digoxigenin (DIG) according to the manufacturer's instructions (DIG RNA labeling kit, Roche Diagnostics Ltd., Lewes, U.K.). Bone tissue was prepared as described above and 5 μm sections were cut and mounted on polylysine-coated slides. Briefly, slides were deparaffinized in xylene, rehydrated, postfixed in PFA (Sigma Chemical Co.,

Dorset, U.K.), followed by digestion for 15 minutes at 37°C with proteinase K (10 μg/ml in 20 mM Tris-HCL, pH 8, 2 mM $CaCl_2$). After a second fixation in PFA, sections were acetylated with freshly prepared 0.25% v/v acetic anhydride in 0.1 M triethanolamine, pH 8. All sections were prehybridized for 2 h at room temperature in 50% formamide, 1 mg/ml BSA, 0.02% w/v Ficoll, 0.02% polyvinylpyrrolidine, 5× SSC, 250 μg/ml tRNA. Hybridization with a heat-denatured probe was carried out at 55°C overnight in prehybridization solution. Aliquots of 70 μl were applied to each section and covered with parafilm coverslips. After hybridization the tissue sections were washed with a series of washes; once for 5 minutes in 5× SSC at 55°C; 1 h in 0.2× SSC at 55°C, followed by 10 minutes at room temperature in 0.2× SSC. The signal was detected according to manufacturer's instructions using the Nitroblue tetrazolium, 5-bromo-4-chloro-3-indoyl-phosphate substrate solution (Roche Diagnostics Ltd.). Slides were mounted in Aquamount (BDH/Merck, Dorset, U.K.) and coverslips applied.

In situ hybridization of human pagetic bone

A human c-*fos* cDNA probe consisting of the terminal 500 bp of coding sequence and 3′-untranslated sequence was random prime labeled with ^{35}S-dCTP. The hybridization treatments were as previously described.[7] Briefly, these included sequential immersion in 0.2 M HCl (20 minutes); 2× SSC (10 minutes); 10 μg/ml of proteinase K in 50 mM Tris-HCl, pH 7.5 (1 h, 37°C); 0.25% w/v glycine in PBS (2 minutes); and freshly prepared 0.25% v/v acetic anhydride in 0.1 M triethanolamine, pH 8. All sections were prehybridized for 1 h at 37°C in 50% formamide, 1 mg/ml BSA, 0.02% w/v Ficoll, 0.02% polyvinylpyrrolidine, 0.6 M NaCl, 0.2 mg/ml of sheared salmon sperm DNA, 10 mM Tris (pH 7.4), 0.5 M EDTA, 10 mM dithiothreitol (DTT), and 10% w/v dextran sulfate. Hybridization with heat-denatured probe was carried out at 37°C overnight in prehybridization solution. Aliquots of 50 μl were applied to each slide and covered with siliconized coverslips. After hybridization the tissue sections were washed with a series of high stringency washes; twice for 5 minutes in 0.5× SSC with 1 mM EDTA; 15 minutes in 50% formamide, 0.15 M NaCl, 5 mM Tris (pH 7.5), and 0.5 mM EDTA; and four times for 5 minutes in 0.5× SSC at 58°C, followed by 5 minutes at room temperature in 0.5x SSC. Slides were dehydrated in 70% and 95% ethanol with 0.3 M ammonium acetate and air dried. Autoradiography was performed with Ilford K5 emulsion (Ilford Imaging U.K. Ltd., Cheshire, U.K.), melted at 40°C and diluted 1:1 with distilled water. The slides were exposed at 4°C for 14 days and then developed for 5 minutes, rinsed, fixed for 5 minutes, and counterstained with hematoxylin and eosin.

Immunohistochemistry

The c-Fos antibody used in this study (c-Fos; sc52), was purchased from Santa Cruz Laboratories (Santa Cruz, CA, U.S.A.). Bone tissue was prepared as detailed above, and 5 μm sections were cut and mounted on TESPA-treated slides. Slides were deparaffinized in xylene and rehydrated. Immunohistochemistry was performed following standard protocols following pretreatment in 3% H_2O_2 in methanol and rehydration. Briefly, sections were permeabilized in 1% w/v SDS in TBS (pH 8.0) for 5 minutes, rinsed twice in TBS and blocked for 1 h in 50% normal rabbit serum (DAKO, Ely, U.K.) in 2% BSA/TBS. Primary antibodies were used at a dilution of 1:50 in 10% normal rabbit serum in TBST for 1 h and washed 3 × 5 minutes in TBST. A biotinylated goat-anti-rabbit secondary antibody (DAKO) was used at a dilution of 1:300 in TBST for 1 h. Negative controls consisted of either TBS or a nonspecific primary antibody (rabbit anti-FLAG epitope) (Santa Cruz Labs). Proteins were visualized using the ABC system (Vector Labs, Burlingame, CA, U.S.A.) with a DAB substrate (Sigma). Slides were counterstained with hematoxylin and mounted in DePeX (BDH/Merck).

RESULTS

Skeletal abnormalities in TRAP-c-fosLTR transgenic mice

The c-*fos* construct used to generate transgenic mice (TRAP-c-*fos*LTR) contains the murine genomic c-*fos* gene fused to the murine TRAP promoter (Fig. 1). In addition, the 3′ destabilizing sequences and polyadenylation (polyA) site of c-*fos* have been deleted and replaced with a 3′ LTR from the FBJ-MSV; this modification is essential for ensuring stability of the exogenous c-*fos* mRNA.[11,19] This vector gives rise to two exogenous c-*fos* transcripts, a 3.0-kb transcript that terminates at the polyA site in the LTR and which encodes for the functional c-Fos protein, and a shorter 2.0-kb transcript that terminates at a cryptic polyA site present in the FBJ-derived sequence. A total of nine founder animals were obtained that contained between ~5 and 50 copies of the transgene as judged by Southern blot analysis of tail DNA. Of these, five developed noticeable swellings in the long bones between 3 and 6 weeks of age, and some mice were severely growth retarded. Radiographic analysis of all phenotypic founder animals indicated the presence of marked skeletal abnormalities in virtually all bones, in contrast to the normal skeletal development in transgene-negative littermates (Fig. 2). Of the five phenotypic animals, three displayed a severe sclerotic phenotype over the entire skeleton with long bones, vertebrae, pelvis and skull being severely affected (Fig. 2A). In the remaining two founder animals, skeletal lesions were detected that developed rapidly into very large calcified tumors (Fig. 2B). Again, these lesions were present in most bones of the skeleton. All phenotypic mice developed these skeletal abnormalities shortly after birth (3–6 weeks postnatally), prior to reaching sexual maturity, and together with the size and severity of the lesions, attempts to breed them and obtain transgenic offspring were not successful. Nevertheless, the generation of five independent founders that developed similar phenotypes (see below) clearly indicated that the defects were independent of the transgene integration site, and suggested that exogenous c-Fos expression was causal in generating the skeletal abnormalities.

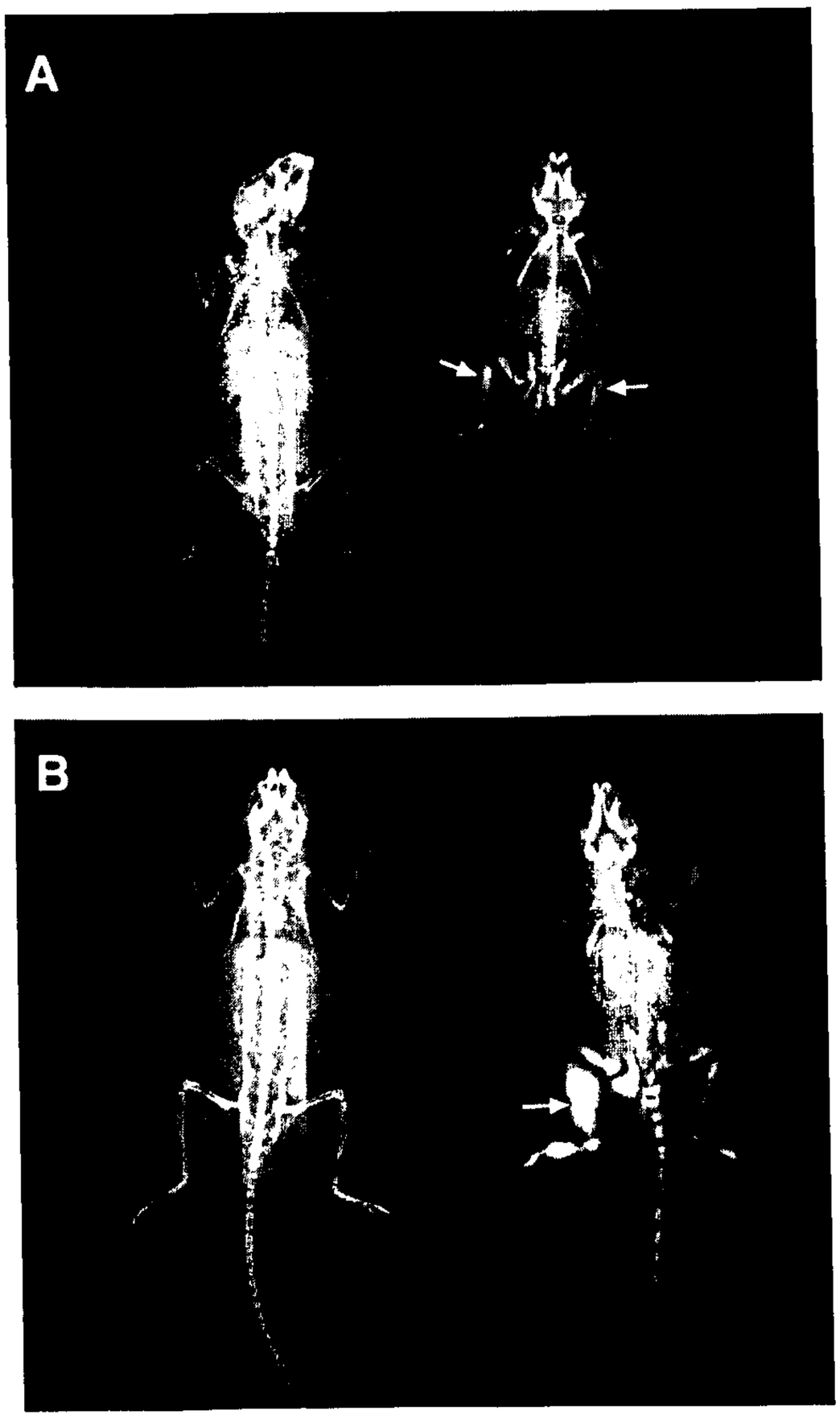

FIG. 2. Skeletal abnormalities in TRAP-c-*fos*LTR transgenic mice. Radiographic analysis of wild-type (left) and transgene positive (right) littermates, showing the severity of lesions in transgenic bone. (A) X-rays of a 5-week-old normal mouse (no. 228–2) and its age-matched transgenic littermate (no. 228–1) showing specific radio-dense lesions in all bones (arrows), including long bones, vertebrae, pelvis, and skull. (B) X-rays of a 5-week-old normal mouse (no. 250–8) and its age-matched transgenic littermate (no. 250–9) showing large calcified tumors (arrow).

*Expression of exogenous c-*fos *in transgenic tissues*

To investigate whether there was a relationship between the development of the skeletal phentoypes in the founder transgenic mice and expression of the c-*fos* transgene, we performed Northern blot analysis on tumor tissues as well as on unaffected tissues. In one particular transgenic founder, exogenous c-*fos* was expressed in tumor-bearing bones and at lower levels in the heart, but not in any other tissues (Fig. 3). Intestine and muscle, and at lower levels brain and liver tissues, also expressed endogenous c-*fos* RNA. In a second independent founder, transgene expression was detected at high levels in lesional bone tissue, but also at lower levels in liver, that did not develop any macroscopic abnormalities (data not shown). Significantly, Northern blot analysis of tissues from the four founder animals that did not develop any bone abnormalities (see above) failed to demonstrate expression of the c-*fos* transgene in tissues analyzed. These data therefore suggest that the expression of exogenous c-*fos* was likely to be responsible for the development of the observed skeletal lesions.

Histologic analysis of skeletal lesions

Histopathologic analysis of sections from representative long bones of TRAP-c-*fos*LTR transgenic mice revealed an increase in bone trabeculae when compared to bones from nontransgenic littermates with a marked bone marrow fibrosis (Figs. 4A and 4B). Long bones in general also exhibited a poorly developed cortical bone (data not shown). Histochemical staining for the osteoclast marker TRAP demonstrated a marked increase in the number of osteoclasts (Fig. 4C) compared to wild-type bones where only a few osteoclasts were observed primarily lining the distal endochondral growth plate (data not shown). The osteoclasts in the transgenic bones were also larger than normal and contained a larger number of nuclei per osteoclast (Figs. 4D and 4E). The lesions appeared to undergo extensive bone remodeling as evidenced by the numerous reversal lines present in all trabeculae (Figs. 4D and 4E). In addition to osteoclasts and osteoblasts, occasional pockets of chondrocytes were observed suggesting that chondrogenic cells or specific precursors were also affected (data not shown). To confirm further the increase in number and activity of osteoclasts within these remodeling lesions, we performed nonradioactive in situ hybridization experiments on adjacent sections of transgenic bones for MMP-9, which is another specific marker for osteoclasts in vivo.[14,19] The results suggested that all TRAP-positive cells were also expressing high levels of MMP-9 (Fig. 4F), which were large and contained numerous nuclei (Figs. 4G and 4H), confirming that these were bona fide osteoclasts.

To investigate the cell-specific expression of c-Fos within these lesions, we performed immunocytochemical analysis on adjacent sections for c-Fos protein using a murine c-Fos polyclonal antibody. The results indicated that high levels of c-Fos protein were detected in almost all osteoclasts lining the bone surfaces (Figs. 4I and 4J). In addition, we observed c-Fos protein expression in some, but not all, osteoblasts and chondrocytes (Figs. 4I and 4J) and in many fibroblastic connective tissue cells within these lesions. It should be emphasized that the antibody we used detects both endogenous as well as exogenous c-Fos proteins, therefore it is perhaps not surprising that many cell types might be expressing c-Fos. Indeed, this is suggested by the Northern blot analyses where endogenous c-*fos* RNA was also detected within the bone lesions (Fig. 3). Taken together, the hallmarks of the phenotype that develops in c-*fos*-overexpressing transgenic mice are an increase in the number and multinuclearity of osteoclast-like cells that result in the development of highly remodeling bone lesions.

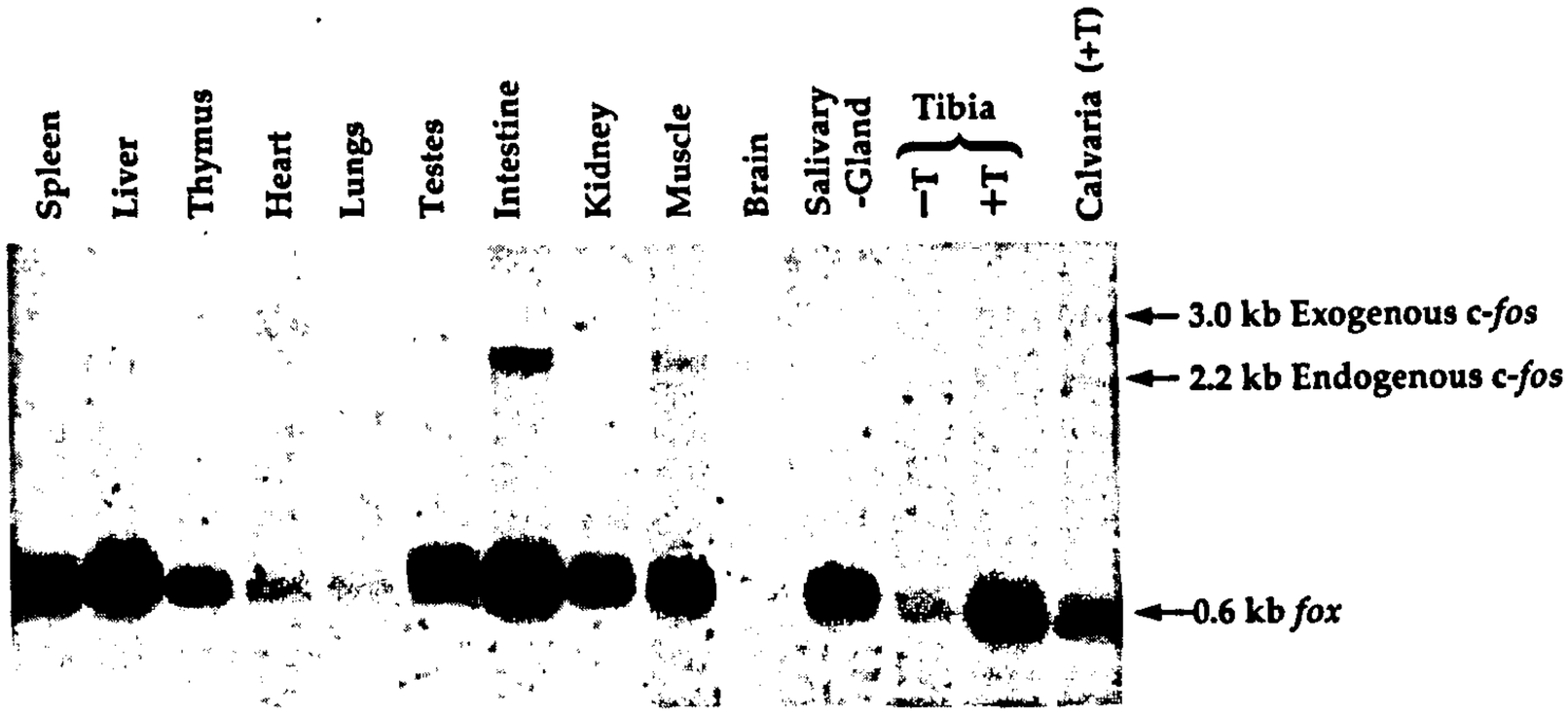

FIG. 3. Northern blot analysis of c-*fos* transgene expression in different tissues of a TRAP-c-*fos*LTR transgenic mouse (no. 1766). The membrane was hybridized with a ^{32}P-labeled v-*fos* probe which detects both exogenous (3.0 kb) and endogenous (2.2 kb) c-*fos* transcripts. The endogenous *fox* gene (0.6 kb) was used as a control for RNA loading. The exogenous c-*fos* transcript was evident in tumor-bearing bones (+ T) like tibia and calvaria, compared with no signal in bones lacking tumors (–T).

*Expression of c-*fos *in bone cells of patients with Paget's disease*

To investigate whether high levels of c-Fos are also present in bone cells of patients with Paget's disease, and to assess cell specificity, we further analyzed the expression of c-*fos* by radioactive in situ hybridization techniques. Using paraffin sections of pagetic bone obtained from biopsies, the results indicated very high c-*fos* expression which was localized to multinucleated osteoclasts (Figs. 5A and 5B), whereas c-*fos* expression in osteoclasts present in control osteophyte tissue was not detectable (see also[7]). In addition, the expression of c-*fos* mRNA was confirmed by immunohistochemical staining for c-Fos protein on adjacent sections (see also[7]) These data further implicate a role for c-Fos in the altered osteoclastic activity which is characteristic of Paget's disease, and confirms previous results using different pagetic bone samples.[7] More importantly, the basic similarities between the morphology and activity of the osteoclasts present in Paget's disease compared with those that develop in TRAP-c-*fos*LTR transgenic mice suggest that these mice might be useful for further delineating the molecular defects underlying the altered osteoclastic behavior, and perhaps also the subsequent events leading to osteosarcoma formation.

DISCUSSION

In this study we have investigated the consequences of ectopic c-Fos expression in mature osteoclasts in an attempt to mimic the high levels of c-Fos that are observed in pagetic osteoclasts.[7] While earlier c-Fos knock-out experiments have demonstrated unequivocally that c-Fos must be expressed in osteoclast progenitors for normal differentiation,[14] it was not possible in that study to discern whether c-Fos is also required for the fusion and activity of fully differentiated, multinucleated osteoclasts. Here, we have shown that targeting c-*fos* expression to osteoclasts under the control of the osteoclast-specific TRAP promoter (TRAP-c-*fos*LTR mice) results in the rapid development of highly remodeling lesions and formation of bone tumors. Five independent transgenic founder mice were identified which expressed exogenous c-Fos but which could not be bred to generate individual families of transgenic mice due to the severity and early onset of the phenotype. Thus, while the absolute causal role of c-Fos in the development of these phenotypes is difficult to prove by analyzing only founder animals, it is clear that the observed skeletal defects were very specific to c-Fos overexpression and independent of the transgene integration site.

The phenotypes among each affected animal, such as the time of onset, the histologic characteristics and cellular composition, were remarkably similar in all bones analyzed from all founder mice, suggesting that the defects were likely caused by ectopic c-Fos expression. Notably, all lesions contained numerous osteoclasts, most of which contained a very large number of nuclei when compared to osteoclasts in wild-type littermates, and in this regard these lesions exhibit some similarities to Paget's disease. The osteoclasts expressed high levels of TRAP activity in addition to MMP-9, which is a reliable specific marker for osteoclasts in vivo. It is tempting to speculate that the consequences of this increased osteoclast number and activity in TRAP-c-*fos*LTR mice are the stimulation of a cascade in which there is an increase in the recruitment and differentiation of osteoblasts, which leads to a highly remodeling lesion in which osteoclastic bone resorption and osteoblastic bone formation are accelerated, causing bone deformation. Despite the fact that the initial appearance of some of the skeletal defects (Figs. 1A and 4B) might suggest an apparent osteopetrotic phenotype implying that osteoclasts are inactive, the presence of abundant reversal lines and continued growth of these lesions suggests that this is not

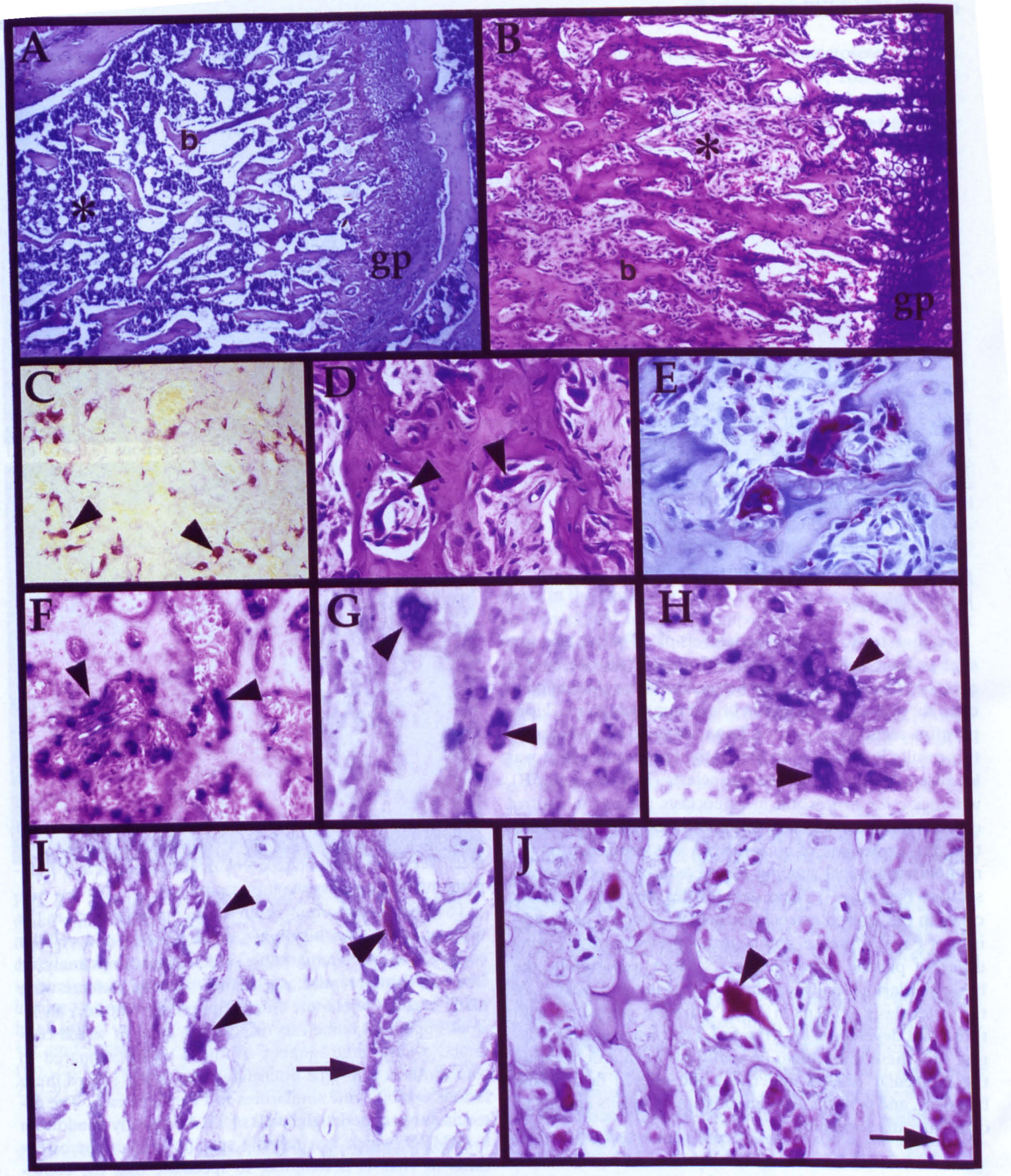

FIG. 4. Histologic analysis of the lesions that develop in TRAP-c-*fos*LTR transgenic mice. Hematoxylin and eosin stained sections of a typical wild-type tibia (A) and a TRAP-c-*fos*LTR transgenic tibia (B), demonstrating the presence of increased bone trabeculae in transgenic mice compared to nontransgenic littermates. The bone marrow space is largely fibrotic with little hematopoietic cell activity (*). TRAP histochemistry and hematoxylin and eosin staining detected increased numbers of osteoclasts (C–E) compared to wild-type bone (data not shown). (C) Detection of TRAP-positive osteoclasts in adjacent sections to above, and (E) a high power view of multinucleated, TRAP-positive osteoclasts (counterstained with hematoxylin). DIG-labeled antisense riboprobes for MMP-9 detected high levels of expression of MMP-9 in TRAP-positive, multinucleated osteoclasts of TRAP-c-*fos*LTR transgenic mice (F–H). Control sense riboprobe hybridizations revealed no specific signal (data not shown). Immunolocalization of c-Fos protein using a murine polyclonal antibody confirms the expression of c-Fos in osteoclasts (I,J). Osteoblasts also express c-Fos in some areas, although clearly negative osteoblasts are also observed (arrow in I). Similarly, some chondrocytes express c-Fos (arrow in J). All sections are 5 μm paraffin sections prepared and stained as described in Materials and Methods. Arrowheads in all panels indicate osteoclasts. gp, growth plate, b, trabecular bone. A,B 100×; C,F x200×; D,E,G–J 400×.

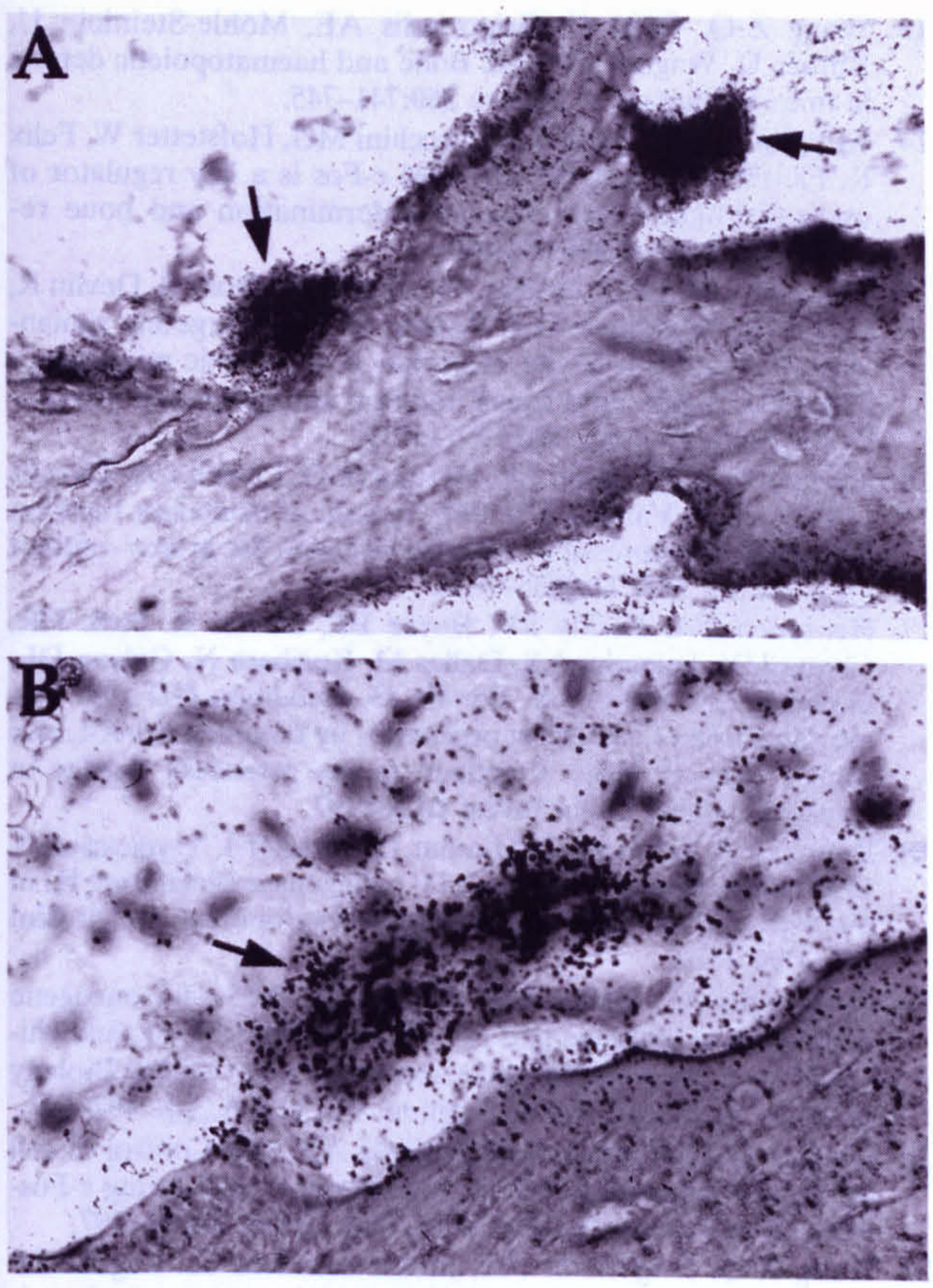

FIG. 5. Expression of c-*fos* in bone cells in Paget's disease. RNA in situ hybridization analysis shows that large multinucleated osteoclasts (arrows) express high levels of c-*fos* (A,B). Hybridization of adjacent sections with a sense c-*fos* probe revealed no specific signal (data not shown). The micrographs are representative of the results obtained from six different Paget's patients (see also[7]).

the case and the osteoclasts are indeed active. This nevertheless remains to be confirmed by examining in greater detail the morphology of the transgenic osteoclasts and by measuring their resorptive activity in vitro. The fact that some of the transgenic mice developed very large bone tumors also suggests that additional events are occurring following expression of the transgene, such as activation of genes that are important in the growth control of specific bone cell populations.[20] While this is a possible scenario, we cannot exclude the possibility that the early lesions would have eventually expanded extracortically to more closely resemble the tumor lesions (Fig. 1B), which we would not have observed because the mice were sacrificed at a relatively early age.

Assessing the possible causes of tumor formation in TRAP-c-*fos*LTR mice is very important in view of the fact that these tumors resemble in some ways the remodeling osteosarcomas that develop in other c-Fos transgenic mice (H2-c-*fos*LTR) that have been generated previously.[10] In H2-c-*fos*LTR mice, chondroblastic osteosarcomas develop due to specific transformation of osteoblastic cells, and osteoblasts express high levels of the transgene. The fact that we have large numbers of osteoblasts (and in some cases chondrocytes) in TRAP-c-*fos*LTR lesions, a large proportion of which express c-Fos protein by immunocytochemical analysis, raises several possible interpretations. First, since the antibody used cannot distinguish between endogenous and exogenous c-Fos, it is possible that expression in osteoblasts reflects endogenous c-Fos protein; efficient expression of the endogenous gene is confirmed by our Northern blot analyses. This may be possible since expression of endogenous c-Fos in osteoclasts is normally very low[20] and activated osteoblasts during bone formation may indeed turn on endogenous c-Fos expression.[10] Second, it is possible that high c-Fos levels in osteoblasts could be interpreted as inappropriate expression of the transgene from the TRAP promoter; that is, we cannot rule out that "leaky" expression of the TRAP promoter in osteoblasts may have caused the observed tumor phenotype. Possible explanations for this scenario might be attributed to the 3′ FBJ-LTR which we have used in our construct. Although it has been demonstrated unequivocally that this fragment is necessary for efficient expression of c-Fos in bone tissue, it cannot be excluded that there exist sequences within this fragment which can override or alter the specificity of the selected promoter. The evidence that this could indeed occur in transgenic mice has previously been discussed.[19,21] To address the issue of target cell specificity, we are currently performing in situ hybridization analyses using a probe that is specific for the transgene (pB15) as described previously,[10] which will determine whether osteoclasts and/or osteoblasts are expressing exogenous c-*fos*.

Taken together, the skeletal abnormalities that develop in TRAP-c-*fos*LTR transgenic founder mice share some properties with Paget's disease, namely, an increase in the number of large, active osteoclasts that express high levels of c-Fos, increased remodeling activity, and cellular transformation and tumor formation. Whether other characteristics are shared, for example, high Bcl-2 levels in osteoclasts,[6] is not known for TRAP-c-*fos*LTR mice, but preliminary evidence suggests that in the remodeling osteosarcomas in H2-c-*fos*LTR mice,[10] high levels of Bcl-2 expression have been observed in osteoclasts (E. El-Emir and A.E. Grigoriadis, unpublished observations). It is very clear that independent families of transgenic mice must be generated to determine unequivocally parameters such as the time of onset of transgene expression, the time of onset of microscopic phenotypic changes, the target cells for transformation in these mice, etc. Furthermore, establishing lines of TRAP-c-*fos*LTR transgenic mice will also provide a useful tool for investigating osteoclast activity in vitro, for example the osteoclastogenic capacity of hematopoietic spleen precursors, or by culturing cells derived from tumors in the presence of receptor activator of NF-KB ligand (RANKL) (also designated tumor necrosis factor-related activation-induced cytokine [TRANCE], osteoprotegerin ligand [OPGL], osteoclast differentiation factor [ODF]),[22–24] which is essential for osteoclast differentiation. It is also likely that immortalized osteoclastic cell lines can be established from these lesions, as has been shown for other oncogenes targeted to osteoclasts.[17] Finally, although TRAP-c-*fos*LTR transgenic mice cannot be consid-

ered as definitive models for Paget's disease, their usefulness will become evident by investigating whether the increased osteoclast activity and remodeling defects can be inhibited using antiresorptive compounds such as bisphosphonates, either in a preventive way, or to treat already formed skeletal lesions. It is becoming very clear that the causes of Paget's disease will be multifactorial. The generation of TRAP-c-*fos*LTR mice will address only one component of the observed abnormalities in Paget's disease, namely, the up-regulation of c-Fos, and will provide a well-defined, readily manipulatable system to test the consequences of altered c-Fos expression in osteoclasts.

ACKNOWLEDGMENTS

We would like to thank Dr. G.D. Roodman for providing the TRAP promoter construct and Dr. N. Faruque for microinjection and generation of transgenic mice. We would also like to thank Dr. J. Hoyland for the in situ hybridization analysis on pagetic bone and Dr. A. Sunters for the c-Fos immunocytochemistry.

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Address reprint requests to:
Dr. Agi E. Grigoriadis
Department of Craniofacial Development
Guy's Hospital
Guy's Tower Floor 28, London Bridge
London SE1 9RT U.K.

Received in original form March 15, 1999; in revised form May 14, 1999; accepted May 27, 1999.